UNITED STATES DISTRICT COURT
SOUTHERN DISTRICT OF NEW YORK

21 CV 9660

X

DIEMIRUAYA OGHENEAKPOR DENIRAN,
3155 ROCHAMBEAU AVENUE,
#9D,
BRONX, NEW YORK, 10467

Plaintiff (s),

Vs.

MUSEUM OF MODERN ART ("MOMA" OR "MUSEUM")
11 West 53rd street
New York, NY 10019
CAROLINE CLEMENTS
11 West 53rd street
New York, NY 10019

Defendant(s).

X

Civil Action No.

CIVIL COMPLAINT
FOR DECLARATORY
JUDGMENT AND INJUNCTIVE
RELIEF

JURY TRIAL DEMANDED

CIVIL COMPLAINT

Plaintiff, Diemiruaya Ogheneakpor Deniran (hereinafter "Deniran") , appearing Pro Se, hereby complains and alleges the following:

INTRODUCTORY STATEMENT

1. This is an action seeking declaratory judgment and injunctive relief against defendants for their violation of established Federal Statutes as outlined herein. This Court has jurisdiction over this case pursuant to 28 U.S.C. §§ 1331 and 1343(a)(3)-(4) (equitable relief), and 42 U.S.C. §§ 1983 and 1988, as well as under non statutory equitable

jurisdiction. The violations alleged are related to the novel coronavirus SARS-CoV-2, which the World Health Organization said can cause the disease COVID-19, which had spread across the globe as of the Spring of 2020. Plaintiff alleges and maintains the position that the premises relied upon by the defendants to terminate his employment of 9 years was a clear violation of the EUA statute, 21 U.S.C. § 360bbb-3, and in so doing have concurrently violated the letter of 42 U.S.C. §§ 1983 and 1988.

JURISDICTION

2. This Court has jurisdiction over the causes of action in this case pursuant to 28 U.S.C. §§ 1331 and1343(a)(3)-(4) (equitable relief), and 42 U.S.C. §§ 1983 and 1988, as well as under non-statutory equitable jurisdiction. The claims in this complaint arise under the Constitution and statutes of the United States and because Deniran seeks prospective redress against state actors in their official capacity to end the deprivation, under state law, of his rights, privileges, and immunities secured by federal law.

3. VENUE

4. Venue for this action properly lies in this District pursuant to 28 U.S.C. § 1391 because Deniran resides in this judicial district and a substantial part of the events, actions, or omissions giving rise to the claim occurred in this judicial district, where defendants MOMA and CAROLINE CLEMENTS are principally located.

5. Upon information and belief, this Court is vested with equitable powers to issue nonstatutory injunctions to protect Plaintiff, Deniran against wayward state actors engaged

in unlawful conduct. *See Trump v. Vance*, 140 S. Ct. 2412, 2428-29 (2020) ("*Ex parte Young*, 209 U.S. 123, 155–156 (1908) (holding that federal courts may enjoin state officials to conform their conduct to federal law).")

6. Plaintiff alleges that defendants MOMA AND CAROLINE CLEMENTS possess a firsthand indomitable connection and control to the enforcement of MOMA'S COVID-19 vaccine mandate and as such are proper actors within the framework as outlined in paragraph 5 above.

7. Defendants respectively run MOMA, administer it, control its activities, have authority to bind it, and personally participated in formulating and issuing the mandate being challenged here *See generally Free Enter. Fund v. PCAOB*, 561 U.S. 477, 491 n.2 (2010) (collecting cases in the vein of *Bell v. Hood*, 327 U.S. 678, 684 (1946) ("[I]t is established practice for this Court to sustain the *jurisdiction* of federal courts to issue injunctions to protect rights safeguarded by the Constitution")

8. PARTIES IN THIS ACTION

9. Plaintiff , Diemiruaya Ogheneakpor Deniran, a United States Citizen, was hired by defendant, MOMA, on August 8, 2012, as a security officer. He remained in that position for 16 months and was promoted to Control Room Operator-assistant supervisor based on his excellent work ethic and record with MOMA until his services were unlawfully and improperly terminated on October 21, 2021.

10. Defendant, Museum of Modern Art ("MoMA" or "Museum"), upon information and belief is chartered as an educational institution whose collection of modern and contemporary art is made available to its members and the public to encourage an ever-deeper understanding and enjoyment of such art by the diverse local, national, and international audiences that it serves.

11. Defendant, CAROLINE CLEMENTS, acted in the capacity of MOMA'S BENEFITS MANAGER, on the day of October 12, 2021, and was responsible for issuing the termination letter to Plaintiff. She is sued here in her official and personal capacities.

12. FACTUAL ALLEGATION

13. Plaintiff, Diemiruaya Ogheneakpor Deniran, was hired by defendant, MOMA, on August 8, 2012, as a security officer. He remained in that position for 16 months and was promoted to Control Room Operator-assistant supervisor based on his excellent work ethic and record with MOMA until his services were unlawfully and improperly terminated on October 21, 2021.

14. All through the 9 years Plaintiff functioned as an employee of defendants, he has maintained an exemplary and excellent record of work ethic, efficiency, and productivity backed up with very good evaluations.

15. As a result of which he received promotion as Control Room Operator-assistant supervisor and efficiently carried out all assigned duties.

16. His compliance, efficiency and work ethic was never an issue with defendants since he began working there.

17. On or about the Spring of 2020, the world became aware that a certain novel corona-virus SARS-CoV-2, which can cause the disease COVID-19, had spread across the globe.

18. Upon Information and belief, the Federal Government launched "Operation Warp Speed," which aimed at developing three separate corona-virus vaccines for the United States for emergency use against COVID-19 which had been declared a global pandemic by the World Health Organization.

19. Because of the Federal government's "Operation Warp Speed," three separate corona-virus vaccines were developed and approved more swiftly than any other vaccine in our nation's history.

20. Upon information and belief, The Food and Drug Administration ("FDA") issued an Emergency Use Authorization ("EUA") for the Pfizer- BioNTech COVID-19 Vaccine ("Pfizer Vaccine") on December 11, 2020.

21. One week later, the FDA issued a second EUA for the Moderna COVID-19 Vaccine ("Moderna Vaccine")

22. Upon information and belief, other vaccines have since been added to the list of FDA'S EUA list.

23. The Plaintiff respectfully now brings to the Court's attention the letter of the statute that the EUA vaccines were authorized under, to wit, 21 U.S.C. § 360bbb-3.

24. It is extremely noteworthy that Plaintiff should respectfully display the letter of the statute which the FDA relied on to approve into the Public domain the EUA vaccines being complained of in this action because it is germane to Plaintiff's contention that defendants violated the law by mandating the vaccination without an option to refuse as commanded by the EUA STATUTE.

25. The EUA statute, 21 U.S.C. § 360bbb-3, explicitly states that anyone to whom the product is administered must be informed of the option to accept or to refuse it, as well as the alternatives to the product and the risks and benefits of receiving it.

26. Upon information and belief, on or about July 2021, The Mayor of the city of New York issued an order for mandatory covid-19 testing for all City Municipal workers.

27. Shortly after, on or about August 3, 2021, upon information and belief, a mandatory proof of the EUA vaccination was required for all in door places of business in the City of New York, including defendants place of business located at 11 West 53rd Street, New York, NY.

28. Defendant, MOMA, upon information and belief, on their own accord and volition adopted the CITYOF NEW YORK EUA VACCINE MANDATE, and implemented a mandatory policy as a condition for Plaintiff retaining his employment with MOMA.

29. Plaintiff, when confronted by defendant CLEMENTS, demanding that he produce proof of vaccination, sent to her and the human resources series of notices informing them that the

EUA statute, 21 U.S.C. § 360bbb-3, explicitly states that anyone to whom the product is administered must be informed of the option to accept or to refuse it.

30. Defendants ignored the entire contents of the notices and the many United States Supreme Court cases which supported his position and instead issued threats of immediate job loss to plaintiff if he did not show them proof of having receiving the vaccine shots.

31. Plaintiff reminded them through of the notices that defendants will irreparably damage his substantive due process rights if they continued with their threats of job termination, but they simply ignored him and eventually terminated his employment on October 21, 2021. [Please see attached proof of job termination]

32. CLAIMS OF DAMAGES & TORTIOUS INTERFERENCE AGAINST DEFENDANTS

33. Plaintiff made diligent effort to convey the available facts of the potency and established reliability on natural immunity to defendants but they capriciously ignored him .

34. For example, upon information and belief and relying on the most consensus scientific opinion on the subject matter, it is well settled that there is currently more data on the durability of natural immunity than there is for vaccine immunity, researchers rely on the expected durability of natural immunity to predict that of vaccine immunity. [Please see detailed scientific study published by Drs. Daniela Weiskopf, Alessandro Sette, and Shane Crotty from the La Jolla Institute for Immunology , and posted on the website of National Institute of Health (NIH) titled, "*Lasting immunity found after recovery from COVID-19"*

dated January 26, 2021, accessed through the Internet via this URL, "https://www.nih.gov/news-events/nih-research-matters/lasting-immunity-found-after-recovery-covid-19" article attached herein as exhibit A.

35. In the above cited study, the researchers concluded in pertinent part that, "*Several months ago, our studies showed that natural infection induced a strong response, and this study now shows that the responses last," Weiskopf says. "We are hopeful that a similar pattern of responses lasting over time will also emerge for the vaccine-induced responses*." [Emphasis is by Plaintiff]

36. As illustrated in the foregoing paragraphs, the Policy of forced vaccine mandate as promoted and enforced by defendants, MOMA, constitutes an unconstitutional condition, because it is poorly disseminated to protect the public health, yet it poses disproportionate risks on some of its targets.

37. As such, with facts demonstrated herein, such Mandate remains an unlawful condition insufficiently germane to its purported purpose. Furthermore, the disciplinary and other draconian burdens that MOMA is using to leverage ostensibly voluntary compliance with its Policy are not proportional to the purported public health aims.

38. The above cited ambiguity remains in conflict with the objectives of the statute governing administration of medical products authorized for emergency use only. Pursuant to the Supremacy Clause of the United States Constitution, federal law overrides conflicting state

law and as such any STATE LAW relied upon by defendants MOMA in this matter is preempted and must be enjoined.

39. Plaintiff, Deniran, HAS EXPERIENCED, AND WILL CONTINUE TO EXPERIENCE, CONCRETE AND PARTICULARIZED HARM AS A DIRECT CONSEQUENCE OF MOMA'S VACCINE POLICY.

CLAIMS FOR RELIEF

COUNT I: VIOLATION OF THE RIGHT TO REFUSE UNWANTED AND MEDICALLY UNNECESSARY MEDICAL CARE

40. Plaintiff realleges and incorporates by reference the foregoing allegations as if fully set

41. MOMA'S coercive Policy requires Deniran to take a vaccine without his consent—and against the expert medical advice of his immunologist—thereby depriving him of his ability to refuse unwanted medical care.

42. Defendants cannot show that they have a compelling interest in coercing Plaintiff, Deniran into taking a COVID-19 vaccine, because GMU has no compelling interest in treating employees with natural immunity any differently from employees who obtained immunity from a vaccine.

43. Plaintiff Deniran, has suffered and will continue to suffer damage from Defendants' conduct. There is no adequate remedy at law, as there are no remedies that could compensate Deniran for the deprivation of his constitutional rights. He will suffer irreparable harm unless this Court enjoins Defendants from enforcing their Policy.

44. COUNT II: VIOLATION OF THE UNCONSTITUTIONAL CONDITIONS DOCTRINE AND THE FOURTEENTH AMENDMENT'S RIGHT TO DUE PROCESS.

45. Plaintiff realleges and incorporates by reference the foregoing allegations as if fully set

46. Unconstitutional conditions case law often references the existence of varying degrees of coercion. According to that body of law, MOMA cannot impair Deniran's right to refuse medical care through subtle forms of coercion any more than it could through an explicit mandate. *See, e.g.*, *Koontz v. St. Johns River Water Mgmt. Dist.*, 570 U.S. 595 (2013)

47. ("[U]nconstitutional conditions doctrine forbids burdening the Constitution's enumerated rights by coercively withholding benefits from those who exercise them"); *Memorial Hosp. v. Maricopa Cty.*, 415 U.S. 250 (1974) ("[An] overarching principle, known as the unconstitutional conditions doctrine … vindicates the Constitution's enumerated rights by preventing the government from coercing people into giving them up").

48. The Due Process Clause of the Fourteenth Amendment provides: "nor shall any state deprive any person of life, liberty, or property, without due process of law … ." U.S. Const., amend. XIV, sec. 1.

49. Plaintiff, Deniran, possesses both a liberty interest in his bodily integrity and, as a Professional, a property interest in his remunerative employment career.

50. MOMA cannot by means of its Policy reverse the burden of proof and require Deniran to prove that it is safe for him to work without being vaccinated. And setting up such a process, which is what MOMA's Policy does, thereby represents a concurrent *procedural*

due process violation and an unconstitutional condition burdening his liberty interests to be free of unwanted medical interventions.

COUNT III—VIOLATION OF THE SUPREMACY CLAUSE

51. Plaintiff realleges and incorporates by reference the foregoing allegations as if fully set

52. Defendants' Policy requires and mandates Deniran to receive a vaccine in order to work effectively without regard to his natural immunity or the health risks he faces.

53. He also must divulge personal medical information by uploading it into an online portal and is threatened with termination action if he declines to comply with these arbitrary mandates.

54. The Policy thus coerces or, at the very least, unduly pressures Deniran into getting a vaccine that FDA approved only for emergency use.

55. The United States Constitution and federal laws are the "Supreme Law of the Land" and supersede the constitutions and laws of any state. U.S. Const. art. VI, cl. 2.

56. "State law is pre-empted to the extent that it actually conflicts with federal law."*English v. General Elec. Co.*, 496 U.S. 72, 79 (1990) (internal citations and quotation marks omitted).

57. Federal law need not contain an express statement of intent to preempt state law for a court to find any conflicting state action invalid under the Supremacy Clause. *See Geier v. American Honda*, 520 U.S. 861, 867-68 (2000).

58. Federal law preempts any state law that creates "an obstacle to the accomplishment and execution of the full purposes and objectives of Congress." *Arizona v. United States*, 567 U.S. 387, 399-400 (2012).

59. The EUA statute mandates informed and voluntary consent. *See John Doe No. 1 v. Rumsfeld*, No. Civ. A. 03-707(EGS), 2005 WL 1124589, *1 (D.D.C. Apr. 6, 2005) (allowing use of anthrax vaccine pursuant to EUA "on a *voluntary* basis"). *See also* 21 U.S.C. § 360bbb- 3(e)(1)(A)(ii).

60. It expressly states that recipients of products approved for use under it be informed of the "option to accept or refuse administration," and of the "significant known and potential benefits and risks of such use, and of the extent to which such benefits and risks are unknown."

61. Since MOMA's Policy (a state program) coerces Deniran by making enjoyment of his constitutionally and statutorily protected consent rights contingent upon receiving an experimental vaccine, it cannot be reconciled with the letter or spirit of the EUA statute. *See* 21 U.S.C. § 360bbb-3.

62. The conflict between MOMA'S State Policy and the EUA statute is very lucid given that the statute's informed consent language requires that recipients be given the "option to refuse" the EUA product. That is at odds with the Policy's forcing Deniran to sustain significant irreparable injury to his career through his sudden termination for refusing to take the vaccine.

63. Moma's Policy clearly frustrates the objective of the EUA statute.

64. Deniran has suffered and will continue to suffer damage from Defendants' conduct. There is no adequate remedy at law, as there are no damages that could compensate Deniran for the deprivation of his constitutional or statutory rights.

65. Defendants' Policy is invalid pursuant to Article VI, Cl. 2 of the United States Constitution, and must be enjoined and set aside.

66. 42 U.S.C. § 1983 provides a civil right of action for deprivations of constitutional protections taken under color of law.

67. Deniran is entitled to declaratory and injunctive relief pursuant to 42 U.S.C. § 1983 because he is being deprived of "rights, privileges, or immunities secured by the Constitution and laws." Section 1983 thus supports both Deniran's constitutional and statutory causes of action against the MOMA defendants because Section 1983 protects rights "secured by the Constitution *and* laws." 42 U.S.C. § 1983

RELIEF REQUESTED

WHEREFORE, Plaintiff , Diemiruaya Ogheneakpor Deniran, respectfully requests that the Court find the Defendants have committed the violations alleged and described above,

A. A declaratory judgment that MOMA's Policy infringes upon Plaintiff's constitutionally protected rights to protect his bodily integrity and to refuse unnecessary medical treatment.

B. Award Compensatory and punitive damages, that may inadequately provide financial compensation to Plaintiff for the sudden termination of his employment and career; including but

not limited to accumulated benefits estimated to be in excess of $10 million US Dollars in lieu of his pension and retirement which have suddenly vanished under the color of law, in tandem with his reasonable life expectancy, productivity and skill, as sole provider for his family and household.

Plaintiffs also seek monetary damages against all defendants of $20 million US dollars.

C. Injunctive relief restraining and enjoining Defendants, their officers, agents, servants, employees, attorneys, and all persons in active concert or participation with them (*see* Fed. R. Civ. P. 65(d)(2)), and each of them, from enforcing coercive or otherwise pressuring policies or conditions similar to those in the Policy that act to compel or try to exert leverage on MOMA's employees with natural immunity to get a COVID-19 vaccine.

D. An award of reasonable Attorneys Fees

E. Any and all further relief this Honorable Court may deem fair and equitable.

JURY DEMAND

Plaintiff herein demands a trial by jury of any triable issues in the present matter.

Respectfully Submitted,

November 18, 2021.

Diemiruaya Ogheneakpor Deniran
3155 Rochambeau Avenue,
#9D
BRONX, NEW YORK, 10467

DEFENDANTS:
MUSEUM OF MODERN ART ("MOMA" OR "MUSEUM")
11 West 53rd street
New York, NY 10019

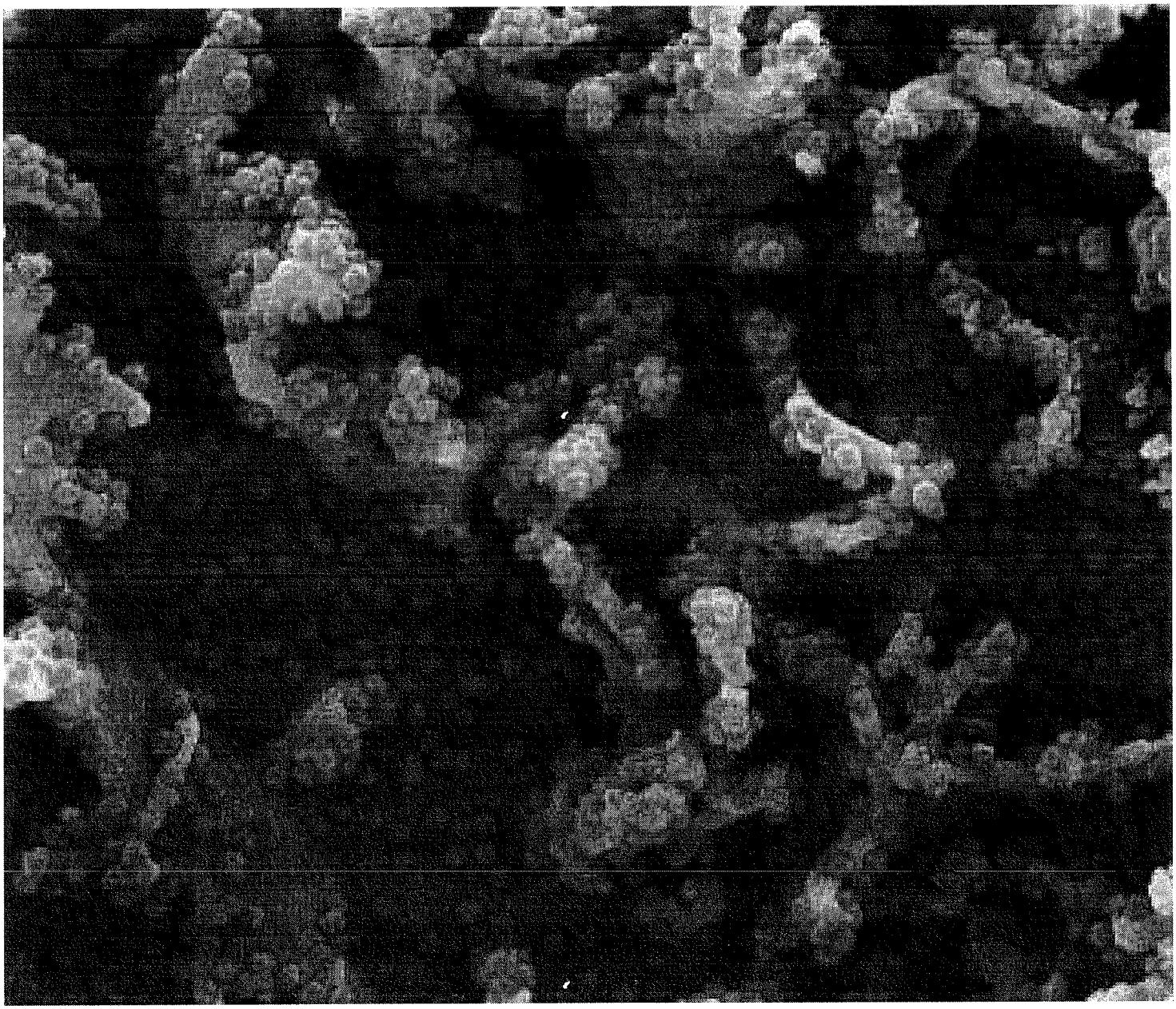
CAROLINE CLEMENTS
11 West 53rd street
New York, NY 10019

January 26, 2021

Lasting immunity found after recovery from COVID-19

At a Glance

- The immune systems of more than 95% of people who recovered from COVID-19 had durable memories of the virus up to eight months after infection.
- The results provide hope that people receiving SARS-CoV-2 vaccines will develop similar lasting immune memories after vaccination.



Colorized scanning electron micrograph of a cell, isolated from a patient sample, that is heavily infected with SARS-CoV-2 virus particles (red). *NIAID Integrated Research Facility, Fort Detrick, Maryland*

After people recover from infection with a virus, the immune system retains a memory of it. Immune cells and proteins that circulate in the body can recognize and kill the pathogen if it's encountered again, protecting against disease and reducing illness severity.

This long-term immune protection involves several components. Antibodies—proteins that circulate in the blood—recognize foreign substances like viruses and neutralize them. Different types of T cells help recognize and kill pathogens. B cells make new antibodies when the body needs them.

All of these immune-system components have been found in people who recover from SARS-CoV-2, the virus that causes COVID-19. But the details of this immune response and how long it lasts after infection have been unclear. Scattered reports of reinfection with SARS-CoV-2 have raised concerns that the immune response to the virus might not be durable.

To better understand immune memory of SARS-CoV-2, researchers led by Drs. Daniela Weiskopf, Alessandro Sette, and Shane Crotty from the La Jolla Institute for Immunology analyzed immune cells and antibodies from almost 200 people who had been exposed to SARS-CoV-2

and recovered.

Time since infection ranged from six days after symptom onset to eight months later. More than 40 participants had been recovered for more than six months before the study began. About 50 people provided blood samples at more than one time after infection.

The research was funded in part by NIH's National Institute of Allergy and Infectious Diseases (NIAID) and National Cancer Institute (NCI). Results were published on January 6, 2021, in *Science*.

The researchers found durable immune responses in the majority of people studied. Antibodies against the spike protein of SARS-CoV-2, which the virus uses to get inside cells, were found in 98% of participants one month after symptom onset. As seen in previous studies, the number of antibodies ranged widely between individuals. But, promisingly, their levels remained fairly stable over time, declining only modestly at 6 to 8 months after infection.

Virus-specific B cells increased over time. People had more memory B cells six months after symptom onset than at one month afterwards. Although the number of these cells appeared to reach a plateau after a few months, levels didn't decline over the period studied.

Levels of T cells for the virus also remained high after infection. Six months after symptom onset, 92% of participants had CD4+ T cells that recognized the virus. These cells help coordinate the immune response. About half the participants had CD8+ T cells, which kill cells that are infected by the virus.

As with antibodies, the numbers of different immune cell types varied substantially between individuals. Neither gender nor differences in disease severity could account for this variability. However, 95% of the people had at least 3 out of 5 immune-system components that could recognize SARS-CoV-2 up to 8 months after infection.

"Several months ago, our studies showed that natural infection induced a strong response, and this study now shows that the responses last," Weiskopf says. "We are hopeful that a similar pattern of responses lasting over time will also emerge for the vaccine-induced responses."

—by Sharon Reynolds

Related Links

- Experimental Coronavirus Vaccine Highly Effective (https://www.nih.gov/news-events/nih-research-matters/experimental-coronavirus-vaccine-highly-effective)
- Antibodies and T Cells Protect Against SARS-CoV-2 (https://www.nih.gov/news-events/nih-research-matters/antibodies-t-cells-protect-against-sars-cov-2)
- Immune Cells for Common Cold May Recognize SARS-CoV-2 (https://www.nih.gov/news-events/nih-research-matters/immune-cells-common-cold-may-recognize-sars-cov-2)
- Potent Neutralizing Antibodies Target New Regions of Coronavirus Spike (https://www.nih.gov/news-events/nih-research-matters/potent-neutralizing-antibodies-target-new-regions-coronavirus-spike)
- Potent Antibodies Found in People Recovered from COVID-19 (https://www.nih.gov/news-events/nih-research-matters/potent-antibodies-found-people-recovered-covid-19)
- Novel Coronavirus Structure Reveals Targets for Vaccines and Treatments (https://www.nih.gov/news-events/nih-research-matters/novel-coronavirus-structure-reveals-targets-vaccines-treatments)
- Coronavirus (COVID-19) (https://covid19.nih.gov/)
- Coronavirus Prevention Network (https://www.coronaviruspreventionnetwork.org/)

- Coronavirus (COVID-19) (https://www.coronavirus.gov/)

References: Immunological memory to SARS-CoV-2 assessed for up to 8 months after infection. Dan JM, Mateus J, Kato Y, Hastie KM, Yu ED, Faliti CE, Grifoni A, Ramirez SI, Haupt S, Frazier A, Nakao C, Rayaprolu V, Rawlings SA, Peters B, Krammer F, Simon V, Saphire EO, Smith DM, Weiskopf D, Sette A, Crotty S. *Science*. 2021 Jan 6:eabf4063. doi: 10.1126/science.abf4063. Online ahead of print. PMID: 33408181.

Funding: NIH's National Institute of Allergy and Infectious Diseases (NIAID) and National Cancer Institute (NCI); La Jolla Institute for Immunology; John and Mary Tu Foundation; Bill and Melinda Gates Foundation; Mastercard; Wellcome; Emergent Ventures; Collaborative Influenza Vaccine Innovation Centers; JPB Foundation; Cohen Foundation; Open Philanthropy Project.

In this Edition

Physician-pharmacist collaboration may improve care for opioid addiction

Acute heart transplant rejection detected earlier with new test

Lasting immunity found after recovery from COVID-19

Search NIH Research Matters

Connect with Us

Subscribe to get NIH Research Matters by email

RSS Feed

Facebook

Email us

Mailing Address:
NIH Research Matters
Bldg. 31, Rm. 5B52, MSC 2094
Bethesda, MD 20892-2094

Popular Stories

Common drug may have potential for treating Alzheimer's disease

Moderna COVID-19 booster may protect against variants

Moderna COVID-19 vaccine generates long-lasting immune memory

Autoimmune response found in many with COVID-19

Understanding SARS-CoV-2 antibody binding

About NIH Research Matters

Editor: Harrison Wein, Ph.D. **Assistant Editor:** Erin Bryant

NIH Research Matters is a weekly update of NIH research highlights reviewed by NIH's experts. It's published by the Office of Communications and Public Liaison in the NIH Office of the Director.

ISSN 2375-9593

NIH...Turning Discovery Into Health®

National Institutes of Health, 9000 Rockville Pike, Bethesda, Maryland 20892

U.S. Department of Health and Human Services

PROOF OF SERVICE

SENDER: COMPLETE THIS SECTION

- Complete items 1, 2, and 3.
- Print your name and address on the reverse so that we can return the card to you.
- Attach this card to the back of the mailpiece, or on the front if space permits.

1. Article Addressed to:

Ashley Murphy-Rutski
Benefit Coordinator
MoMA
11 West 53rd street
Newyork, NY 10019

9590 9402 6623 1028 1397 77

2. Article Number (Transfer from service label)

7021 0350 0000 0278 1574

PS Form 3811, July 2020 PSN 7530-02-000-9053

COMPLETE THIS SECTION ON DELIVERY

A. Signature
X Covid-19 ☐ Agent ☐ Addressee

B. Received by (Printed Name) C. Date of Delivery
10/04/21

D. Is delivery address different from item 1? ☐ Yes
If YES, enter delivery address below: ☐ No

3. Service Type
☐ Adult Signature
☐ Adult Signature Restricted Delivery
☑ Certified Mail®
☐ Certified Mail Restricted Delivery
☐ Collect on Delivery
☐ Collect on Delivery Restricted Delivery
☐ Insured Mail
☐ Insured Mail Restricted Delivery (over $500)
☐ Priority Mail Express®
☐ Registered Mail™
☐ Registered Mail Restricted Delivery
☐ Signature Confirmation™
☐ Signature Confirmation Restricted Delivery

Domestic Return Receipt

SENDER: COMPLETE THIS SECTION

- Complete items 1, 2, and 3.
- Print your name and address on the reverse so that we can return the card to you.
- Attach this card to the back of the mailpiece, or on the front if space permits.

1. Article Addressed to:

CAROLINE CLEMENTS
BENEFITS MANAGER
MOMA.
11 WEST 53rd street
New York, NY 10019

9590 9402 6623 1028 1412 99

2. Article Number (Transfer from service label)

7020 2450 0001 2422 3130

PS Form 3811, July 2020 PSN 7530-02-000-9053

COMPLETE THIS SECTION ON DELIVERY

A. Signature
X Covid-19 ☐ Agent ☐ Addressee

B. Received by (Printed Name) C. Date of Delivery
09/23/21

D. Is delivery address different from item 1? ☐ Yes
If YES, enter delivery address below: ☐ No

3. Service Type
☐ Adult Signature
☐ Adult Signature Restricted Delivery
☑ Certified Mail®
☐ Certified Mail Restricted Delivery
☐ Collect on Delivery
☐ Collect on Delivery Restricted Delivery
☐ Insured Mail
☐ Insured Mail Restricted Delivery (over $500)
☐ Priority Mail Express®
☐ Registered Mail™
☐ Registered Mail Restricted Delivery
☐ Signature Confirmation™
☐ Signature Confirmation Restricted Delivery

Domestic Return Receipt

7020 2450 0001 2422 3130

ASHLEY MURPHY-RUTSKI
11 WEST 53rd STREET
NEW YORK, NY 10019

CERTIFIED MAIL® RECEIPT
Domestic Mail Only

$3.75
$2.78
$0.00
$6.53

09/10/2021

SENDER: COMPLETE THIS SECTION

- Complete items 1, 2, and 3.
- Print your name and address on the reverse so that we can return the card to you.
- Attach this card to the back of the mailpiece, or on the front if space permits.

1. Article Addressed to:

JAMES GROOMS
General Counsel & Secretary
MOMA
11 West 53rd Street
New York, NY 10019

9590 9402 6623 1028 1397 84

2. Article Number (Transfer from service label)

7021 0350 0000 0278 1567

PS Form 3811, July 2020 PSN 7530-02-000-9053

COMPLETE THIS SECTION ON DELIVERY

A. Signature
X Covid-19 ☐ Agent ☐ Addressee

B. Received by (Printed Name) C. Date of Delivery
10/04/21

D. Is delivery address different from item 1? ☐ Yes
If YES, enter delivery address below: ☐ No

3. Service Type
☐ Adult Signature
☐ Adult Signature Restricted Delivery
☑ Certified Mail®
☐ Certified Mail Restricted Delivery
☐ Collect on Delivery
☐ Collect on Delivery Restricted Delivery
☐ Insured Mail
☐ Insured Mail Restricted Delivery (over $500)
☐ Priority Mail Express®
☐ Registered Mail™
☐ Registered Mail Restricted Delivery
☐ Signature Confirmation™
☐ Signature Confirmation Restricted Delivery

Domestic Return Receipt

USPS Tracking®

FAQs >

Track Another Package +

Tracking Number: 70210350000002781567

Remove ×

Your item was delivered to the front desk, reception area, or mail room at 10:56 am on October 4, 2021 in NEW YORK, NY 10019.

Delivered, Front Desk/Reception/Mail Room

October 4, 2021 at 10:56 am
NEW YORK, NY 10019

Get Updates ∨

Text & Email Updates ∧

Select what types of updates you'd like to receive and how. Send me a notification for:

Text	Email	
☐	☐	All Below Updates
☐	☐	Expected Delivery Updates ⓘ
☐	☐	Day of Delivery Updates ⓘ
☐	☐	Package Delivered ⓘ

☐ ☐ Available for Pickup ⓘ

☐ ☐ Delivery Exception Updates ⓘ

☐ ☐ Package In-Transit Updates ⓘ

Tracking History

October 4, 2021, 10:56 am
Delivered, Front Desk/Reception/Mail Room
NEW YORK, NY 10019
Your item was delivered to the front desk, reception area, or mail room at 10:56 am on October 4, 2021 in NEW YORK, NY 10019.

October 4, 2021, 8:34 am
Out for Delivery
NEW YORK, NY 10019

October 4, 2021, 8:23 am
Arrived at Post Office
NEW YORK, NY 10019

October 3, 2021
In Transit to Next Facility

October 2, 2021, 9:37 pm
Departed USPS Regional Facility
NEW YORK NY DISTRIBUTION CENTER

October 1, 2021, 9:33 pm
Arrived at USPS Regional Facility
NEW YORK NY DISTRIBUTION CENTER

October 1, 2021, 3:48 pm
USPS in possession of item
BRONX, NY 10467

Product Information

Postal Product:	Features:	See tracking for related item: 9590940266231028139784 (/go/TrackConfirmAction?tLabels=9590940266231028139784)
First-Class Mail®	Certified Mail™	

See Less

Can't find what you're looking for?

Go to our FAQs section to find answers to your tracking questions.

FAQs

USPS Tracking®

FAQs >

Track Another Package +

Remove ×

Tracking Number: 70210350000002781574

Your item was delivered to the front desk, reception area, or mail room at 10:56 am on October 4, 2021 in NEW YORK, NY 10019.

Delivered, Front Desk/Reception/Mail Room

October 4, 2021 at 10:56 am
NEW YORK, NY 10019

Get Updates

Text & Email Updates

Select what types of updates you'd like to receive and how. Send me a notification for:

Text	Email	
☐	☐	All Below Updates
☐	☐	Expected Delivery Updates ⓘ
☐	☐	Day of Delivery Updates ⓘ
☐	☐	Package Delivered ⓘ

☐ ☐ Available for Pickup ⓘ

☐ ☐ Delivery Exception Updates ⓘ

☐ ☐ Package In-Transit Updates ⓘ

Tracking History

October 4, 2021, 10:56 am
Delivered, Front Desk/Reception/Mail Room
NEW YORK, NY 10019
Your item was delivered to the front desk, reception area, or mail room at 10:56 am on October 4, 2021 in NEW YORK, NY 10019.

October 4, 2021, 8:34 am
Out for Delivery
NEW YORK, NY 10019

October 4, 2021, 8:23 am
Arrived at Post Office
NEW YORK, NY 10019

October 3, 2021
In Transit to Next Facility

October 2, 2021, 9:25 pm
Departed USPS Regional Facility
NEW YORK NY DISTRIBUTION CENTER

October 1, 2021, 9:37 pm
Arrived at USPS Regional Facility
NEW YORK NY DISTRIBUTION CENTER

October 1, 2021, 3:50 pm
USPS in possession of item
BRONX, NY 10467

Product Information

Postal Product:	Features:	See tracking for related item: 9590940266231028139777 (/go/TrackConfirmAction?tLabels=9590940266231028139777)
First-Class Mail®	Certified Mail™	

See Less

Can't find what you're looking for?

Go to our FAQs section to find answers to your tracking questions.

FAQs

USPS Tracking®

FAQs >

Track Another Package +

Tracking Number: 70202450000124223130

Remove ×

Your item was delivered to the front desk, reception area, or mail room at 11:20 am on September 13, 2021 in NEW YORK, NY 10019.

Delivered, Front Desk/Reception/Mail Room

September 13, 2021 at 11:20 am
NEW YORK, NY 10019

Get Updates ∨

Text & Email Updates ∧

Select what types of updates you'd like to receive and how. Send me a notification for:

Text	Email	
☐	☐	All Below Updates
☐	☐	Expected Delivery Updates ⓘ
☐	☐	Day of Delivery Updates ⓘ
☐	☐	Package Delivered ⓘ

- [ ] Available for Pickup ⓘ
- [ ] Delivery Exception Updates ⓘ
- [ ] Package In-Transit Updates ⓘ

Tracking History

September 13, 2021, 11:20 am
Delivered, Front Desk/Reception/Mail Room
NEW YORK, NY 10019
Your item was delivered to the front desk, reception area, or mail room at 11:20 am on September 13, 2021 in NEW YORK, NY 10019.

September 13, 2021, 9:54 am
Out for Delivery
NEW YORK, NY 10019

September 13, 2021, 9:43 am
Arrived at Post Office
NEW YORK, NY 10019

September 12, 2021
In Transit to Next Facility

September 11, 2021, 8:19 pm
Departed USPS Regional Facility
NEW YORK NY DISTRIBUTION CENTER

September 10, 2021, 11:23 pm
Arrived at USPS Regional Facility
NEW YORK NY DISTRIBUTION CENTER

September 10, 2021, 4:08 pm
Departed Post Office
BRONX, NY 10467

September 10, 2021, 12:25 pm
USPS in possession of item
BRONX, NY 10467

Product Information

Postal Product: First-Class Mail®	**Features:** Certified Mail™	**See tracking for related item: 9590940266231028141299 (/go/TrackConfirmAction?tLabels=9590940266231028141299)**

See Less

Can't find what you're looking for?

Go to our FAQs section to find answers to your tracking questions.

FAQs

USPS Tracking®

FAQs >

Track Another Package +

Tracking Number: 70202450000124223130

Remove ×

Your item was delivered to the front desk, reception area, or mail room at 11:20 am on September 13, 2021 in NEW YORK, NY 10019.

Delivered, Front Desk/Reception/Mail Room

September 13, 2021 at 11:20 am
NEW YORK, NY 10019

Get Updates ∨

Text & Email Updates ∧

Select what types of updates you'd like to receive and how. Send me a notification for:

Text	Email	
☐	☐	All Below Updates
☐	☐	Expected Delivery Updates ⓘ
☐	☐	Day of Delivery Updates ⓘ
☐	☐	Package Delivered ⓘ

☐ ☐ Available for Pickup ⓘ

☐ ☐ Delivery Exception Updates ⓘ

☐ ☐ Package In-Transit Updates ⓘ

Tracking History

September 13, 2021, 11:20 am
Delivered, Front Desk/Reception/Mail Room
NEW YORK, NY 10019
Your item was delivered to the front desk, reception area, or mail room at 11:20 am on September 13, 2021 in NEW YORK, NY 10019.

September 13, 2021, 9:54 am
Out for Delivery
NEW YORK, NY 10019

September 13, 2021, 9:43 am
Arrived at Post Office
NEW YORK, NY 10019

September 12, 2021
In Transit to Next Facility

September 11, 2021, 8:19 pm
Departed USPS Regional Facility
NEW YORK NY DISTRIBUTION CENTER

September 10, 2021, 11:23 pm
Arrived at USPS Regional Facility
NEW YORK NY DISTRIBUTION CENTER

September 10, 2021, 4:08 pm
Departed Post Office
BRONX, NY 10467

September 10, 2021, 12:25 pm
USPS in possession of item
BRONX, NY 10467

Product Information

Postal Product: First-Class Mail®	**Features:** Certified Mail™	**See tracking for related item: 9590940266231028141299 (/go/TrackConfirmAction?tLabels=9590940266231028141299)**

See Less

Can't find what you're looking for?

Go to our FAQs section to find answers to your tracking questions.

FAQs

EXHIBIT A

The Museum of Modern Art

October 21, 2021

Diemiruaya Deniran
3155 Rochambeau Avenue, Apt 9D
Bronx, NY 10467

Dear Diemiruaya,

Per the Key to NYC mandate, all MoMA employees were required to receive their first COVID vaccine by September 10, 2021 and their second by October 8, 2021. You did not receive the first vaccine by September 10 and were placed on unpaid leave.

On October 8, you came to the HR office and showed Ashley Murphy that you had received your first dose on September 27, and that your second was scheduled for October 18. On October 18, you emailed HR and said that you were reluctant to get the second shot, as it is against your religious beliefs. We emailed and called that same morning and told you that in order to keep your job with MoMA, you would have to show proof of having received the second dose of your COVID vaccine by October 20, 2021.

As you did not send us proof of having received your shot by the deadline, your employment with MoMA is terminated effective October 20, 2021. Included with this letter are an exiting procedure memo and the New York State Department of Labor Record of Employment form.

Please contact me at caroline_clements@moma.org or (212) 708-9455 if you have any questions.

Sincerely,

Caroline Clements

Caroline Clements, Benefits Manager

MoMA
Department of Human Resources

To: Diemiruaya Deniran, Security

cc: Daniel Platt, Security

From: Caroline Clements, Human Resources

Date: October 21, 2021

Re: Exiting Procedures and Benefits Information

In connection with your last day of employment on October 20, 2021, this memorandum will provide you with important information regarding exiting procedures and benefits.

For Manager and Employee

- You are reminded to return all MoMA property to your manager or department head. MoMA property includes:
  - Identification Card
  - Keys
  - Library books
  - Equipment including laptop computer, mobile device, tools, etc.
  - MoMA Chase corporate credit card
  - Uniforms (if applicable)
  - Any other Museum property not mentioned above
- You must reconcile all open balances with the Accounting Department prior to your last day. Contact Susan Inlall at 212-408-8431 with any questions.
- Email and network access will terminate as of close of business on your last day of work

For Manager

Identification Cards

- Please be sure to obtain your employees' identification card and return to Security (Frantz Guillaume).
- If applicable, please be sure to obtain your employee's Chase credit card and return to Nancy Read.

Paychecks

- If you are enrolled in direct deposit, your last check will be paid electronically as usual. If you are not enrolled in direct deposit, your check will be mailed to the home address that HR has on file.
- Any vacation time which you have accrued but not used will be cashed-out. This payment will only be issued after Payroll receives all of the information from the timekeeper in your department. This is usually one to two pay periods after your last day.

Information For Your Records

- Date of Hire: August 8, 2011
- Ending Job Title: Assistant Control Room Supervisor
- Ending Salary: $53,810 per year

Unemployment Insurance

- Attached is the New York State Department of Labor Unemployment Insurance Record of Employment form that we are required by law to provide to all employees who quit, are laid off, or are discharged.
- Receipt of this form does not mean you are eligible for unemployment benefits. Rather it provides certain information we are required to give to employees in compliance with NYS Labor Regulations (12 NYCRR Section 472.8).

Commuter Benefits

- Your commuter benefits through BRI will remain active until the end of the following month of termination to use the funds on your debit card (i.e. if you terminate on 11/10 you have until 12/31 to use those funds).
- After that date, you will receive a new debit card from BRI. They will charge an administrative fee of $5.75 per month against your balance. For additional questions please contact BRI at 800-473-9595 or www.briweb.com.

Statement of Earnings

- Your W-2 form will be mailed to you from the Payroll Department the January following your termination date.
- If your address changes after your termination date, please email your change to human_resources@moma.org or complete the attached Address Change form and send to the Human Resources Department at The Museum of Modern Art, 11 West 53rd Street, New York, NY 10019.

Exit Interview

If you would like to set up an exit interview with Human Resources to discuss the information in this memo or your experiences at MoMA, please contact a member of the HR Team.

NEW YORK STATE OF OPPORTUNITY | Department of Labor

Unemployment Insurance Division

Record of Employment

(For Unemployment Insurance purposes only.)

Employer: Complete the following and give this form to every employee who quits, is laid off, or is discharged:

Date given to employee: 10/22/2021

NYS Employer Registration No.: 04-5068 04

Federal Employer Identification No.: 13-1624100

Employer Name: The Museum of Modern Art

Payroll Records are kept at: 11 West 53rd Street

Street: 11 West 53rd Street

Street:

City: New York State: NY Zip: 10019

Optional if needed by employer to locate employee record:

Payroll or Clock No.: _____ Location of employment or code: _____

Employee: Keep this certificate. Have it with you if you apply for Unemployment Insurance (UI) benefits. This certificate shows that your job was insured. It does not necessarily mean you qualify for benefits. The UI Claims Center will make that determination if you apply. Please complete the following:

Your Name: Diemiruaya Deniran Social Security No.: ___-___-____

This may not be used as an identification card.

IA 12.3 (01/18)

How to Apply For New York State Unemployment Insurance

Unemployment Insurance is temporary income for eligible workers who are out of work through no fault of their own. It provides them a weekly benefit while they look for work. If you become unemployed and want to apply for Unemployment Insurance benefits, apply online at www.labor.ny.gov for a quick and convenient way to file your claim or call the Telephone Claim Center toll free at (888) 209-8124.

Have the following information available when you apply:

1. Your Social Security Number.
2. Your NYS Driver License or Motor Vehicle ID card (if you have either one).
3. Your complete mailing address and zip code.
4. A phone number, including area code, where we can reach you from 8:00 am – 5:00 pm, Monday – Friday.
5. Your alien registration card (if you are not a US citizen and have a card).
6. Employer information (even employers in other states) for the last 18 months which includes:
   - Employer names, addresses, and phone numbers.
   - NYS Employer Registration Number or Federal Employer Identification Number (FEIN). The FEIN is on your W-2 form(s).
   - Your total gross earnings (before any deductions) for each employer. You may be asked for pay stubs, W-2 forms, or other payment records.
7. Your copies of "Notice to Federal Employee about Unemployment Insurance" (Form SF8) and "Notification of Personnel Action" (Form SF50) if you were a federal employee.
8. Your most recent separation form (DD214) and any DD215 forms you have received from military service. You can request a DD 214 through the U.S. National Archives and Records Administration website at: http://www.archives.gov/st-louis/military-personnel/standard-form-180.html.
9. A check from your personal checking account so you can provide your bank's routing number and your checking account number if you choose Direct Deposit of your weekly benefits.

You can file a claim without all of these documents. However, missing information could delay your first payment.

EXHIBIT B

Diemiruaya Ogheneakpor Deniran
3155 Rochambeau Avenue,
#9D,
Bronx, New York, 10467
9/7/2021

Caroline Clements and Ashley Murphy
Human Resources
Museum of Modern Art ("MoMA" or "Museum")
11 West 53rd street
New York, NY 10019

RE: Mandatory COVID-19 Vaccination Policy

NUNC PRO TUNC CONDITIONAL ACCEPTANCE FOR VALUE FOR PROOF OF CLAIM & irrevocable admission of liability, tender of certified surety related to your enforcement OF *Mandatory COVID-19 Vaccination Policy*, AND FOR DETERMINATION OF 'MEETING OF THE MINDS, AND VERIFICATION OF THE SOURCE OF YOUR AUTHORITY IN LIGHT OF THE SUPREME LAWOF THE LAND AS FOUND IN, Cruzan, 497 U.S. at 278; King v. Rubenstein, 825 F.3d 206, 222 (4th Cir. 2016),

STATE OF NEW YORK):

COUNTY OF NEW YORK):

Diemiruaya Deniran now says as follows:

1. We are in receipt of your NOTICE, identified as, *Mandatory COVID-19 Vaccination Policy* .

2. SINCE THE SAID NOTICE HAS DRASTIC PUNITIVE MEASURES THAT DIRECTLY & IMMEDIATELY AFFECTS MY SOURCE OF

LIVELIHOOD, AND DUE TO OUR FINDINGS THAT THE SUPREME LAWS OF THE LAND SO NOT SUPPORT YOUR MANDATE, WE THEREFORE DEMAND THAT YOU RESPOND TO THE FOLLOWING INTERROGATORIES/PROOF OF CLAIM DESIGNED TO EXCULPATE YOU ANY AND ALL LIABILITIES DERIVABLE FROM YOUR DESIRE TO IMPLEMENT THIS IMPERATIVE:

3. **PLEASE TAKE NOTICE** THAT YOUR REFUSAL TO provide the demanded responses will be proof that you intended to be deemed a tort feasor in your capacity as a *state actor* as defined in 42 U.S. Code § 1983, including but not limited to other liabilities as found in TITLE 18, U.S.C., SECTION 242.

4. As I want to resolve this matter as soon as possible, I am initiating this private-administrative remedy to determine such unresolved issues of material fact which is predicated upon your return TO US WITHIN 48 HOURS THE PROOFS DEMANDED HEREIN:

5. The necessary **'Proofs of Claim'** are set out below, to wit:

1. PROOF OF CLAIM DEMAND:

2. DENY OR ADMIT THAT Museum of Modern Art ("MoMA" or "Museum") IN IMPLEMENTING THE ALLEGED MANDATE ARE ACTING AS AGENTS OF CITY OF NEW YORK?

3. IF YOU ANSWERED YES TO DEMAND NO 2: YOU MUST RETURN CERTIFIED AUTHORITY precluding you from acting UNDER COLOR OF LAW OF AUTHORITY?

6. DENY OR ADMIT THAT YOU LACK KNOWLEDGE OF THE FOLLOWING UNITED STATES SUPREME COURT DECISIONS REGARDING THE SUBJECT OF ONE'S unalienable right to REFUSE unwanted medical care, and as stated above, YOUR REFUSAL TO provide the demanded responses will be proof that you intended to be deemed a tort feasor in your capacity as a *state actor* as defined in 42 U.S. Code § 1983, including but not limited to other liabilities as found in TITLE 18, U.S.C., SECTION 242.

7. The Supreme Court has recognized that the Ninth and Fourteenth Amendments protect an individual's right to privacy. A "forcible injection … into a non-consenting person's body represents a substantial interference with that person's liberty[.]" *Washington v. Harper*, 494 U.S. 210, 229 (1990).

8. 'At common law, even the touching of one person by another without consent and without legal justification was a battery'). See, e.g., Cruzan v. Dir., Mo. Dep't of Public Health, 497 U.S. 261, 278 (1990)

9. ARE YOU AWARE THAT, THE US CONSTITUTION, protects a person's right to "refus[e] unwanted medical care." Cruzan, 497 U.S. at 278; King v. Rubenstein, 825 F.3d 206, 222 (4th Cir. 2016), AND AS SUCH, pursuant to the Supreme Doctrine found in "*The claim and exercise of a constitutional right cannot be converted into a crime.*" *Miller v. US*, 230 F 486, at 489.]

10. This right is "so rooted in our history, tradition, and practice as to require special protection under the Fourteenth Amendment." Washington v. Glucksberg, 521 U.S. 702, 722 n.17 (1997).

11. The Court has explained that the right to refuse medical care derives from the "wellestablished, traditional rights to bodily integrity and freedom from unwanted touching." Vacco v. Quill, 521 U.S. 793, 807 (1997).

12. Coercing employees to receive an EUA vaccine for a virus that presents a near-zero risk of illness or death to them and which they are exceedingly unlikely to pass on to others, because those employees already possess natural immunity to the virus, violates the liberty and privacy interests

that the Ninth and Fourteenth Amendments protect. See, *Washington v. Harper*, 494 U.S. 210, 229 (1990).

13. When a state policy implicates a fundamental right, through coercion or otherwise, the strict scrutiny standard "applies[:] a law will not be upheld unless the government demonstrates that the law is necessary to further a compelling governmental interest and has been narrowly tailored to achieve that interest." Mohamed v. Holder, 266 F. Supp. 3d 868, 877 (E.D. Va. 2017)

14. Similarly, the United States requires everyone, including its citizens, to provide proof of a negative COVID-19 test before returning to the country from abroad. Documentation of recovery suffices as a substitute, although proof of vaccination does not. See Requirement of Proof of Negative COVID-19 Test or Recovery from COVID-19 for All Air Passengers Arriving in the United States, CDC (July 6, 2021), available at https://bit.ly/3yfcJDM (last visited July 28, 2021).

15. Unconstitutional conditions case law often references the existence of varying degrees of coercion. According to that body of law, MOMA cannot impair Diemiruaya Déniran's right to refuse medical care through subtle forms of coercion any more than it could through an explicit

mandate. See, e.g., *Koontz v. St. Johns River Water Mgmt. Dist.*, 570 U.S. 595 (2013)

16. ("[U]nconstitutional conditions doctrine forbids burdening the Constitution's enumerated rights by coercively withholding benefits from those who exercise them"); Memorial Hosp. v. Maricopa Cty., 415 U.S. 250 (1974) ("[An] overarching principle, known as the unconstitutional conditions doctrine … vindicates the Constitution's enumerated rights by preventing the government from coercing people into giving them up").

17. The government "may not deny a benefit to a person on a basis that infringes his constitutionally protected interests"); *Wieman v. Updegraff*, 344 U.S. 183, 192 (1952)

18. The United States Constitution and federal laws are the "Supreme Law of the Land" and supersede the constitutions and laws of any state. U.S. Const. art. VI. cl. 2.

19. "State law is pre-empted to the extent that it actually conflicts with federal law." English v. General Elec. Co., 496 U.S. 72, 79 (1990)

20. **PROOF OF CLAIM** that **CITY OF NEW YORK**, as an **'artificial entity/creature,'** created under the laws of the State of NEW YORK and doing business in the State of NEW YORK, by and through its Officers,

Board of Directors and employees, and agents are not bound to support Article VI, CLAUSE 2, THE SUPREMACY CLAUSE.

General acquiescence or non-response by Museum of Modern Art ("MoMA" or "Museum") to provide the above **'Proofs of Claim'** will constitute your agreement and formal acceptance. You will have by your non-response FAILED to state a claim upon which relief can be granted otherwise shall operate as general acquiescence relative to this presentment. You will have admitted there is no valid Claim of Action arising FROM YOUR INTENTION TO IMPLEMENT a coercive, intrusive and unconstitutional MANDATE, and in so doing you admit liability and surety to the affiant IF YOU INDEED CARRY out your threat of forcible termination of his current employment.

Due to the time sensitive nature of this private matter, under necessity, you are to respond with **'Proof of Claim'** within 3 days, plus three (3) days grace granted by return service by certified-priority-return-mail to the undersigned's address .

Should you fail or refuse by non-response to provide **'Proof of Claim'** within the time specified in this private matter, general acquiescence and acceptance will be taken on your part as formally exercised (performed) pursuant to your silence.

This agreement shall have the affect of an instrument under seal.

Sincerely,

Without Prejudice

Authorized Representative, Attorney-In-Fact

By: _____

Diemiruaya Ogheneakpor Deniran
Secured Party Creditor

NEW YORK NOTARY PUBLIC

HARUNUR RASHID
Notary Public, State of New York
Bronx County, # 01RA6114955
My Commission Expires
August 30, 2024

MY COMMISSION EXPIRES

United States Patent **7,279,327**
Curtis , et al. **October 9, 2007**

**Please see images for: (Certificate of Correction) **

Methods for producing recombinant coronavirus

Abstract

A helper cell for producing an infectious, replication defective, coronavirus (or more generally nidovirus) particle cell comprises (a) a nidovirus permissive cell; (b) a nidovirus replicon RNA comprising the nidovirus packaging signal and a heterologous RNA sequence, wherein the replicon RNA further lacks a sequence encoding at least one nidovirus structural protein; and (c) at least one separate helper RNA encoding the at least one structural protein absent from the replicon RNA, the helper RNA(s) lacking the nidovirus packaging signal. The combined expression of the replicon RNA and the helper RNA in the nidovirus permissive cell produces an assembled nidovirus particle which comprises the heterologous RNA sequence, is able to infect a cell, and is unable to complete viral replication in the absence of the helper RNA due to the absence of the structural protein coding sequence in the packaged replicon. Compositions for use in making such helper cells, along with viral particles produced from such cells, compositions of such viral particles, and methods of making and using such viral particles, are also disclosed.

Inventors:	**Curtis; Kristopher M.** (Chapel Hill, NC), **Yount; Boyd** (Hillsborough, NC), **Baric; Ralph S.** (Haw River, NC)
Assignee:	**The University of North Carolina at Chapel Hill** (Chapel Hill, NC)
Family ID:	**26963129**
Appl. No.:	**10/474,962**
Filed:	**April 19, 2002**
PCT Filed:	**April 19, 2002**
PCT No.:	**PCT/US02/12453**
371(c)(1),(2),(4) Date:	**May 25, 2004**
PCT Pub. No.:	**WO02/086068**
PCT Pub. Date:	**October 31, 2002**

Prior Publication Data

Document Identifier	Publication Date
US 20040235132 A1	Nov 25, 2004

Related U.S. Patent Documents

Application Number	Filing Date	Patent Number	Issue Date
60285320	Apr 20, 2001		
60285318	Apr 20, 2001		

Current U.S. Class:	**435/325**; 435/320.1; 424/221.1
Current CPC Class:	C07K 14/005 (20130101); C12N 7/00 (20130101); C12N 15/10 (20130101); C12N 15/86 (20130101); A61K 2039/525 (20130101); C12N 2830/30 (20130101); C12N 2770/20022 (20130101); C12N 2770/20043 (20130101); C12N 2770/20052 (20130101); C12N 2770/20062 (20130101)
Current International Class:	C12N 15/85 (20060101); C12N 15/63 (20060101)
Field of Search:	;435/320.1,325

Other References

Godeke et al. J. Virol. 2000, vol. 74, pp. 1566-1571. cited by examiner .
Izeta et al. J. Virol. Feb. 1999, vol. 73, No. 2, pp. 1535-1545. cited by examiner .
Fisher et al. J. Virol. 1997, vol. 71, No. 7, pp. 5148-5160. cited by examiner .
Goldeke, G.A., Assembly of Spike into Coronavirus Particles is Mediated by the Carboxy-Terminal Domain of the Spike Protein, J. Virol., Feb. 2000, vol. 74, No. 3:1566-1517. cited by other .
Yount, B., Strategy for Systematic Assembly of Large RNA and DNA Genomes: Transmissible Gastroentritis Virus Model, J. Virol., Nov. 2000, vol. 74, No. 22:10600-10611. cited by other .
Curtis, K.M., Heterologous Gene Expression from Transmissible Gastroenteritis Virus Replicon Particles, J.Virol., Feb. 2002, vol. 76, No. 3:1422-1434. cited by other .
Ortego, J., Generation of a Replication-Competent, Propagation-Defecient Virus Vector based on the Transmissible Gastroenteritis Coronavirus Genome, J. Virol., Nov. 2002, vol. 76, No. 22: 11518-11529. cited by other .
International Search Report, PCT/US02/12453, Mar. 13, 2003. cited by other .
Baudoux et al. "Coronavirus Pseudoparticles Formed with Recombinant M and E Proteins Induce Alpha Interferon Synthesis by Leukocytes" Journal of Virology 72(11):8636-8643 (1998). cited by other .
Bos et al. "The Production of Recombinant Infectious DI-Particles of a Murine Coronavirus in the Absence of Helper Virus" Virology 218:52-60 (1996). cited by other .
de Haan et al. "Coronavirus Particle Assembly: Primary Structure Requirements of the Membrane Protein" Journal of Virology 72(8):6838-6850 (1998). cited by other .
Fuerst et al. "Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase" Proc. Natl. Acad. Sci. USA 83:8122-8126 (1986). cited by other .
Vennema et al. "Intracellular Transport of Recombinant Coronavirus Spike Proteins: Implications for Virus Assembly" Journal of Virology 64(1):339-346 (1990). cited by other .
Vennema et al. "Nucleocapsid-independent assembly of coronavirus-like particles by co-expression of viral envelope protein genes" The EMBO Journal 15(8):2020-2028 (1996). cited by other .
Pushko et al. "Replicon-Helper Systems from Attenuated Venezuelan Equine Encephalitis Virus: Expression of Heterologous Genes in Vitro and Immunization against Heterologous Pathogens in Vivo" Virology 239:389-401 (1997). cited by other .
Schutz-Cherry et al. "Influenza (A/HK/156/97) Hemagglutinin Expressed by an Alphavirus Replicon System Protects Chickens against Lethal Infection with Hong Kong-Origin H5N1 Viruses" Virology 279:55-59 (2000). cited by other .
Hevey et al. "Marburg Virus Vaccines Based upon Alphavirus Replicons Protect Guinea Pigs and

Nonhuman Primates" 251:28-37 (1998). cited by other .
Percy et al. "A Poliovirus Replicon Containing the Chloramphenicol Acetyltransferase Gene Can Be Used To Study the Replication and Encapsidation of Poliovirus RNA" Journal of Virology 66(8):5040-5046 (1992). cited by other .
Messerte et al. "Cloning and mutagenesis of a herpesvirus genome as an infectious bacterial artificial chromosome" Proc. Natl. Acad. Sci. USA 94:14759-14763 (1997). cited by other .
Khromykh et al. "Subgenomic Replicons of the Flavivirus Kunjin: Construction and Applications" Journal of Virology 71(2):1497-1505 (1997). cited by other .
Fischer et al. "Analysis of Constructed E Gene Mutants of Mouse Hepatitis Virus Confirms a Pivotal Role for E Protein in Coronavirus Assembly" Journal of Virology 72(10);7885-7894 (1998). cited by other .
Fischer et al. "Analysis of a Recombinant Mouse Hepatitis Virus Expressing a Foreign Gene Reveals a Novel Aspect of Coronavirus Transcription" Journal of Virology 71(7):5148-5160 (1997). cited by other .
Dubensky et al. "Sindbis Virus DNA-Based Expression Vectors: Utility for In Vitro and In Vivo Gene Transfer" Journal of Virology 70(1):508-519 (1996). cited by other .
DiCiommo et al. "Rapid, High Level Protein Production Using DNA-based Semliki Forest Virus Vectors" The Journal of Biological Chemistry 273(29):18060-18066 (1998). cited by other .
Caley et al. "Humoral, Mucosal, and Cellular Immunity in Response to a Human Immunodeficiency Virus Type 1 Immunogen Expressed by a Venezuelan Equine Encephalitis Virus Vaccine Vector" Journal of Virology 71(4):3031-3038 (1997). cited by other .
Bredenbeek et al. "Sindbis Virus Expression Vectors: Packaging of RNA Replicons by Using Defective Helper RNAs" Journal of Virology 67(11):6439-6446 (1993). cited by other .
Almazan et al. "Engineering the largest RNA virus genome as an infectious bacterial artificial chromosome" PNAS 97(10):5516-5521 (2000). cited by other .
Agapov et al. "Noncytopathic Sindbis virus RNA vectors for heterlogous gene expression" Proc. Natl. Acad. Sci. USA 98:12989-12994 (1998). cited by other .
Meulenberg et al. "Infectious Transcripts from Cloned Genome-Length cDNA of Porcine Reproductive and Respiratory Syndrome Virus" Journal of Virology 72(1):380-387 (1998). cited by other .
Curtis et al. "Reverse Genetic Analysis of the Transcription Regulatory Sequence of the Coronavirus Transmissible Gastroenteritis Virus" Journal of Virology 78(11):6061-6066 (2004). cited by other .
Yount et al. "Reverse genetics with a full-length infectious cDNA of severe acute respirator syndrome coronavirus" PNAS 100(22):12995-13000 (2003). cited by other .
Vamavski et al. "Stable High-Level Expression of Heterologous Genes In Vitro and in Vivo by Noncytopathic DNA-Based Kunjin Virus Replicon Vectors" Journal of Virology 74(9):4394-4403 (2000). cited by other .
Porter et al. "Encapsidation of Genetically Engineered Poliovirus Minireplicons Which Express Human Immunodeficiency Virus Type 1 Gag and Pol Proteins upon Infection" Journal of Virology 67(7):3712-3719 (1993). cited by other .
Balasuriya et al. "Expression of the Two Major Envelope Proteins of Equine Arteritis Virus as a Heterodimer Is Necessary for Induction of Neutralizing Antibodies in Mice Immunized with Recombinant Venezuelan Equine Encephalitis Virus Replicon Particles" Journal of Virology 74(22):10623-10630 (2000). cited by other .
Berglund et al. "Enhancing immune responses using suicidal DNA vaccines" Nature Biotechnology 16:562-565 (1998). cited by other .
Liljestrom et al. "A New Generation of Animal Cell Expression Vectors Based On The Semliki Forest Virus Replicon" Biotechnology 9:1356-1361 (1991). cited by other .
Dollenmaier et al. "Membrane-Associated Respiratory Syncytial Virus F Protein Expressed from a Human Rhinovirus Type 14 Vector Is Immunogenic" Virology 281:216-230 (2001). cited by other .
Mendez et al. "Molecular Characterization of Transmissible Gastroenteritis Coronavirus Defective Interfering Genomes: Packaging and Heterogeneity" Virology 217:495-507 (1996). cited by other .
Curtis et al. "A Simple Strategy to Assemble Infectious RNA and DNA Clones" Adv. Exp. Med. Biol. 494:475-481 (2001). cited by other .

Curtis et al. "Coronavirus Dervied Vectors for Genetic Analysis and Heterologous Gene Expression" Recent Res. Devel. Virol. 4:203-229 (2002). cited by other .
Khromykh et al. "Replicon-based vectors of positive strand RNA viruses" Current Opinion in Molecular Therapeutics 2(5):555-569 (2000). cited by other .
Yount et al. "Biological consequences of TGEV Gene Rearrangement" 20.sup.th Annual American Society for Virology Meeting, Madison, WI, Jul. 21-25, 2001 (Abstract). cited by other .
Yount et al. "Coronavirus Heterologous Gene Expression Vectors" Sixth Int'l Symposium on Positive Strand RNA viruses, Institute Pasteur, Paris France, May 28-Jun. 2, 2001 (Abstract). cited by other .
Yount et al. "Rewiring the severe acute respiratory syndrome coronavirus (SARS-CoV) transcription circuit: Engineering a recombination-resistant genome" PNAS 103(33):12546-12551 (2006). cited by other.

Primary Examiner: Campell; Bruce R.
Assistant Examiner: Li; Bao Quh
Attorney, Agent or Firm: Myers, Bigel, Sibley & Sajovec, P.A.

Government Interests

STATEMENT OF FEDERAL SUPPORT

This invention was made possible with government support under grant numbers AI23946 and GM63228 from the National Institutes of Health. The United States government has certain rights to this invention.

Parent Case Text

RELATED APPLICATIONS

This application claims priority under 35 U.S.C. .sctn. 371 from PCT Application No. PCT/US02/12453, filed in English on Apr. 19, 2002, which claims the benefit of U.S. Application Ser. No. 60/285,320 and U.S. Application Ser. No. 60/285,318, both filed on Apr. 20, 2001, the disclosures and contents of which are incorporated by reference herein in their entireties.

Claims

What is claimed is:

1. A helper cell for producing an infectious, replication defective, coronavirus particle, wherein said cell is a coronavirus permissive cell, comprising: (a) a coronavirus replicon RNA comprising a coronavirus packaging signal, and a heterologous RNA sequence, wherein said replicon RNA further lacks a sequence encoding at least one coronavirus structural protein; and (b) at least one separate helper RNA encoding the at least one structural protein absent from the replicon RNA, said helper RNA lacking a coronavirus packaging signal. wherein the combined expression of the replicon RNA and the helper RNA produces an assembled coronavirus particle which comprises said heterologous RNA sequence, is able to infect a cell, and is replication defective.

2. The helper cell according to claim 1, said replicon RNA further comprising a sequence encoding at least one of the coronavinis structural proteins.

3. The helper cell according to claim 1, wherein said helper RNA contains at least one gene encoding a structural protein selected from the group consisting of the E, M, N, and S genes.

4. The helper cell according to claim 1, wherein said helper RNA contains the E gene.

5. The helper cell according to claim 1, wherein said coronavirus is selected from the group consisting of human respiratory coronavirus, mouse hepatitis virus, porcine transmissible gastroenteritis virus, porcine respiratory coronavirus, canine coronavirus, feline enteric coronavirus, feline infectious peritonitis virus, rabbit coronavirus, murine hepatitis virus, sialodacryoadenitis virus, porcine hemagglutinating encephaloinyclitis virus, bovine coronavirus, avian infectious bronchitis virus, and flu-key coronavirus.

6. The helper cell according to claim 1, wherein said coronavirus is transmissible gastroenteritis virus.

7. The helper cell according to claim 1, wherein said replicon RNA contains at least one attenuating gene order rearrangement among the 3A, 3B, HP, S, E, M and N genes.

8. The helper cell according to claim 1, wherein said helper RNA includes a promoter.

9. The helper cell according to claim 1, wherein said replicon RNA includes a promoter.

10. The helper cell according to claim 1, wherein said heterologous RNA is selected from the group consisting of RNA encoding proteins and RNA encoding peptides.

11. The helper cell according to claim 1, further comprising a heterologous DNA encoding said helper RNA.

12. The helper cell according to claim 1, further comprising a heterologous DNA encoding said replicon RNA.

13. A method of making infectious, replication defective, coronavirus particles, comprising: providing a helper cell according to claim 1; producing said coronavirus particles in said helper cell; and then collecting said coronavirus particles from said helper cell.

14. The method according to claim 13, wherein said coronavinis RNA and said at least one separate helper RNA are introduced into said helper cell by electroporation.

Description

FIELD OF THE INVENTION

The present invention relates to methods of producing recombinant nidovirus vectors, particularly coronavirus vectors, and expressing heterologous genes from said vectors.

BACKGROUND OF THE INVENTION

Transmissible gastroenteritis (TGE) is an economically important, acute enteric disease of swine, which is often 100% fatal in newborn piglets (Enjuanes, et al. (1995) Dev. Biol. Stand. 84:145-152; Enjuanes, et al. (1995) Adv. Exp. Med. Biol. 380:197-211; Laude, et al. (1990) Vet. Microbiol. 23:147-154). TGE virus (TGEV), the causative agent of TGE, is a member of the Coronaviridae family and the order Nidovirales. In addition to the Coronaviridae, the order Nidovirales also includes the Arteriviridae family, of which the swine pathogen porcine reproductive and respiratory syndrome virus (PRRSV) is a member (Cavanagh and Horzinek (1993) Arch. Virol. 128:395-396; de Vries, et al. (1997) Semin. Virol. 8:33-47; Siddell, et al. (1983) J. Gen. Virol. 64:761-776). Despite significant size differences (.about.13 to 32 kb), the polycistronic genome organization and regulation of

gene expression from a nested set of subgenomic mRNAs are similar for all members of the order (de Vries, et al. (1997) Semin. Virol. 8:33-47; Snijder and Horzinek (1993) J. Gen. Virol. 74:2305-2316).

TGEV possesses a single-stranded, positive-sense .about.28.5-kb RNA genome enclosed in a helical nucleocapsid structure that is surrounded by an envelope containing three viral proteins, including the S glycoprotein, the membrane (M) glycoprotein and a small envelope (E) protein (Eleouet, et al. (1995) Virology 206:817-822; Enjuanes and van der Zeijst (1995) In: S. G. Siddell (ed.), The Coronaviridae. Plenum Press, New York, N.Y., p. 337-376; Rasschaert and Laude (1987) J. Gen. Virol. 68:1883-1890; Risco, et al. (1996) J. Virol. 70:4773-4777). Remarkably, only the E and M proteins are absolutely required for particle formation, defining a novel model for virion budding (Fischer, et al. (1998) J. Virol. 72:7885-7894; Vennema, et al. (1996) EMBO J. 15:2020-2028). The TGEV genome contains eight large open reading frames (ORFs), which are expressed from full-length or subgenomic-length mRNAs during infection (Eleouet, et al. (1995) Virology 206:817-822; Sethna, et al. (1991) J. Virol. 65:320-325; Sethna, et al. (1989) Proc. Natl. Acad. Sci. USA 86:5626-5630). The 5'-most .about.20 kb contains the replicase genes in two ORFs, 1A and 1B, the latter of which is expressed by ribosomal frameshifting (Almazan, et al. (2000) Proc. Natl. Acad. Sci. USA 97:5516-5521; Eleouet, et al. (1995) Virology 206:817-822). The 3'-most .about.9 kb of the TGEV genome contains the structural genes, each preceded by a highly conserved transcription regulatory element (TSE) [ACTAAAC; SEQ ID NO:1]. The size of the functional TSE is subject to debate, but ranges from .about.7-15+ nucleotides in length when analyzed in recombinant defective interfering RNAs (Enjuanes, et al. (2001) J. Biotechnology 88:183-204; Jeong, et al. (1996) Virology 217:311-322; Krishnan, et al. (1996) Virology 218:400-405; Joo and Makino (1995) J. Virol. 69:3339-3346). In general, TSE length affects the function of individual mutations because longer elements are generally more resistant to "debilitating" mutations (Enjuanes, et al. (2001) J. Biotechnology 88:183-204). As the leader RNA sequence is also defined by a TSE at its 3' end, some degree of base-pairing between the leader RNA and body TSE likely mediate virus transcription of subgenomic RNAs (Baker and Lai (1990) EMBO J. 9:4173-4179; Baric, et al. (1983) J. Virol. 48:633-640; Makino, et al. (1986) Proc. Natl. Acad. Sci. USA 83:4204-4208; Makino, et al. (1991) J. Virol. 65:6031-6041; Siddell, S. G. 1995. The coronaviridae, An introduction. In: The coronaviridae, eds. S. G. Siddell, Plenum Press, New York. pp 1-10). The subgenomic mRNAs are arranged in a co-terminal nested set structure from the 3' end of the genome, and each contains a leader RNA sequence derived from the 5' end of the genome. Although each mRNA is polycistronic, the 5'-most ORF is preferentially translated, necessitating the synthesis of a distinct mRNA species for each ORF (Lai and Cavanagh (1997) Adv. Virus Res. 48:1-100; McGoldrick, et al. (1999) Arch. Virol. 144:763-770; Sethna, et al. (1991) J. Virol. 65:320-325; Sethna, et al. (1989) Proc. Natl. Acad. Sci. USA 86:5626-5630). Both full-length and subgenomic-length negative-strand RNAs are also produced and have been implicated in mRNA synthesis (Baric and Yount (2000) J. Virol. 74:4039-4046; Sawicki and Sawicki (1990) J. Virol. 64:1050-1056; Schaad and Baric (1994) J. Virol. 68:8169-8179; Sethna, et al. (1991) J. Virol. 65:320-325; Sethna, et al. (1989) Proc. Natl. Acad. Sci. USA 86:5626-5630). Subgenomic RNA synthesis occurs by a method of discontinuous transcription, most likely by transcription attenuation during negative-strand synthesis (Baric and Yount (2000) J. Virol. 74:4039-4046; Sawicki and Sawicki (1990) J. Virol. 64:1050-1056).

The coronavirus E and M proteins function in virion assembly and release, which involve the constitutive secretory pathway of infected cells. Coexpression of the E and M proteins results in virus-like particle formation in cells, defining a novel, nucleocapsid-independent mechanism of enveloped-virus assembly (Vennema, et al. (1996) EMBO J. 15:2020-2028). The role of the E protein in virus assembly was further confirmed by reverse genetic analysis using targeted recombination (Fischer, et al. (1998) J. Virol. 72:7885-7894) and the development of TGEV replicon viruses (Curtis, et al. (2002) J. Virol. 76(3):1422-34). The TGEV M protein may serve to initiate the viral particle assembly process through interactions with genomic RNA and nucleoprotein in pre-Golgi compartments (Narayanan, et al. (2000) J. Virol. 74:8127-8134). The precise role of E in the assembly and release of coronavirus particles is not clear. Although an interaction between the E and M proteins has not yet been demonstrated, such an interaction likely occurs and would serve to facilitate the budding of viral particles. Additionally, E protein has been suggested to "pinch off the neck" of the assembled viral particles during the final stages of budding (Vennema, et al. (1996) EMBO J. 15:2020-2028).

PRRSV is endemic in most swine producing countries. Virions are enveloped 45-70 nm particles that contain 5 envelope proteins and an icosahedral nucleocapsid (N), which surrounds a single-stranded positive polarity RNA

genome of about 15 kb (Pancholi, et al. (2000) J. Infect. Dis. 182:18-27; Pirzadeh and Dea (1998) J. Gen. Virol. 79:989-99). The 15 kDa N protein is most abundant and contains common conformational antigenic sites that are conserved of European and North American strains (Pirzadeh and Dea (1998) J. Gen. Virol. 79:989-99). N is likely multimerized to form icosahedral core structures (20-30 nm), which can be observed by EM. The major envelope proteins include a 25 kDa glycoprotein (GP5) and an 18-19 kDa unglycosylated M protein (Eleouet, et al. (1995) Virology 206:817-22; Meulenberg, et al. (1997) Vet. Microbiol. 55:197-202). GP5 (ORF 5) heterogeneity ranges from 50-90% amino acid identity among isolates, contains at least two neutralizing sites, and expression causes apoptosis (Eleouet, et al. (1995) Virology 206:817-22; Pirzadeh, et al. (1998) Can. J. Vet. Res. 62:170-7; Saif (1999) Transmissible gastroenteritis and porcine respiratory coronavirus, p. 295-325. In B. Straw, D'Allaire, S, Mengeling, W L and Taylor, D J (ed.), Diseases of Swine 8th edition. Iowa State University Press, Ames, Iowa; Tresnan, et al. (1996) J. Virol. 70:8669-74). The M protein (ORF6) contains 3 hydrophobic domains and accumulates in the ER of infected cells, where it forms disulfide-linked heterodimers with GP5 and may function in virus assembly (Meng (2000) Vet. Microbiol. 74:309-29). As with equine arterivirus, it is likely that coexpression of M and GP5 are needed for appropriate post-translational modification, folding and function, and for inducing high neutralizing antibody titers (Balasuriya, et al. (2000) J. Virol. 74:10623-30; Eleouet, et al. (1995) Virology 206:817-220).

Live, attenuated PRRSV vaccines causes viremia and may spread to other pigs. DNA immunization with a plasmid encoding GP5 of PRRSV induces specific neutralizing antibodies and reduces viremia and lung pathology in swine following challenge (Risco, et al. (1996) J. Virol. 70:4773-7). Recombinant adenovirus and vaccinia viruses encoding various PRRSV antigens are also being developed with encouraging results (Budzilowicz, et al. (1985) J. Virol. 53:834-40; Tresnan, et al. (1996) J. Virol. 70:8669-74). Several groups have concluded that effective PRRSV recombinant vaccines must induce high neutralizing titers, induce cellular immunity, induce heterotypic immunity and provide protection at mucosal surfaces (Eleouet, et al. (1995) Virology 206:817-22; Meulenberg, et al. (1997) Vet. Microbiol. 55:197-202). Achieving these goals is complicated by the generally low immunogenicity of the PRRSV envelope proteins and high genomic heterogeneity present in field isolates (Meulenberg, et al. (1997) Vet. Microbiol. 55:197-2020). Hence, improved vaccines are needed.

Recently, a simple and rapid approach for systematically assembling a full-length cDNA copy of the TGEV genomic RNA from which infectious transcripts can be produced has been described (Yount, et al. (2000) J. Virol. 74:10600-10611). This approach, as well as that of Almazan et al. ((2000) Proc. Natl. Acad. Sci. USA 97:5516-5521), will facilitate reverse genetic methods that impact all aspects of coronavirology, however, the production of infectious TGEV replicon particles is still limited.

SUMMARY OF THE INVENTION

A first aspect of the present invention is a helper cell for producing an infectious, replication defective, nidovirus particle. The helper cell comprises (a) a nidovirus permissive cell (e.g., a cell permissive of replication but not necessarily infection); (b) a nidovirus replicon RNA comprising the nidovirus packaging signal and a heterologous RNA sequence, wherein the replicon RNA further lacks a sequence encoding at least one nidovirus structural protein (e.g., lacks one, two, three, four or all); and (c) at least one separate helper RNA (e.g., one, two, three, four, etc. separate helper RNAs) encoding the at least one structural protein absent from the replicon RNA, the helper RNA(s) lacking the nidovirus packaging signal. The combined expression of the replicon RNA and the helper RNA in the nidovirus permissive cell produces an assembled nidovirus particle which comprises the heterologous RNA sequence, is able to infect a cell, and is unable to complete viral replication in the absence of the helper RNA due to the absence of the structural protein coding sequence in the packaged replicon.

Example nidoviruses that may be used to carry out the present invention include members of the familes Coronaviridae and Arteriviridae. Currently preferred are the coronaviruses.

In some embodiments, the replicon RNA further comprises a sequence encoding at least one of the nidovirus structural proteins (for example, the M, N, and/or S genes). In other embodiments, the replicon RNA lacks all of the nidovirus structural proteins. Thus in some embodiments, the helper RNA contains (or helper RNAs contain)

at least one gene encoding a structural protein, such as the E, M, N, and/or S genes.

In certain preferred embodiments, the replicon RNA and/or the helper RNA contains at least one attenuating gene order rearrangement among the 3A, 3B, HP, S, E, M and N genes.

In certain embodiments, the helper RNA and/or the replicon RNA may include a promoter(in the helper RNA, to drive expression of the appropriate helper gene or genes; in the replicon RNA, to drive expression of the coronavirus genes or the heterologous genes (which may be driven by the same or a different promoter). In certain embodiments of the invention, the helper cell may include a heterologous DNA encoding the replicon RNA, and/or a heterologous DNA encoding the helper RNA, with the replicon RNA and/or the helper RNA being transcribed from the corresponding DNA in the permissive cell.

A further aspect of the present invention is a method of making infectious, replication defective, nidovirus particles, comprising: providing a helper cell as described above, producing the nidovirus particles in the helper cell; and then collecting the nidovirus particles from the helper cell. The replicon RNA and the at least one separate helper RNA are stably or transiently introduced into the helper cell by any suitable means, such as electroporation of the RNA into the cell, introduction of DNA into the cell as noted above, etc.

A still further aspect of the invention is infectious nidovirus particles containing a heterologous RNA within a replicon RNA as described above. Such particles may be produced by the methods described above. In certain preferred embodiments the present invention provides a composition comprising a population of infectious, replication defective, nidovirus particles, wherein each particle comprises a nidovirus replicon RNA, wherein the replicon RNA comprises a nidovirus packaging signal and one or more heterologous RNA sequences, wherein the replicon RNA further lacks a sequence encoding at least one nidovirus structural protein, and wherein the population contains no detectable replication-competent nidovirus particles as determined by passage on nidovirus permissive cells (e.g., cells permissive of infection and replication) in culture. As previously, the replicon RNA may further comprise a sequence encoding at least one nidovirus structural protein. Also as previously, the replicon RNA may contain at least one attenuating gene order rearrangement among the 3a, 3b, Hp, S, E, M and N genes.

A still further aspect of the present invention is a pharmaceutical formulation comprising infectious nidovirus particles as described above in a pharmaceutically acceptable carrier.

A still further aspect of the present invention is a method of introducing a heterologous RNA into a subject, comprising administering infectious nidovirus particles as described herein to the subject in an amount effective to introduce the heterologous RNA into the subject.

Nidovirus replicon RNAs and helper RNAs as described above, independent of the helper cell, are also an aspect of the present invention. Such RNAs may be provided in a suitable carrier such as an aqueous carrier for introduction into the helper cell as described above.

Further aspects of the present invention include DNAs encoding replicon RNAs or helper RNAs as described above, along with vectors and recombinant constructs carrying or comprising such DNA, all of which may be used to create the helper cells described above, and which may be provided in a suitable carrier such as an aqueous carrier for introduction into a helper cell as described above.

The foregoing and other objects and aspects of the present invention are explained in detail in the specification set forth below.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A shows the organization of TGEV recombinant viruses encoding GFP. TGEV ORF 3A and 3A and a portion of 3B were removed and replaced with GFP under control of the 3A and N TSE.

FIG. 1B shows the sequence organization of GFP in FiGFP2(PflMI) (SEQ ID NO:2). GFP was inserted downstream of the ORF 3A TSE. The TGEV sequence originating at the 3' end of the S gene through the start of the GFP gene is shown, and the important TSEs and restriction sites are labeled.

FIG. 1C shows the organization of TGEV recombinant fragments encoding GFP. FiGFP2(PflMI) was constructed by deleting ORF 3A and inserting GFP with a 5' 20-nt N gene TSE. Subsequent deletions extending from the unique PflMI to the unique AvrII and EcoNI sites generated FiGFP2-AvrII and FiGFP2-EcoNI fragments, respectively.

FIG. 1D depicts the strategy for assembling recombinant TGEV and replicon cDNAs. The six cDNA subclones (TGEV A, B1, B2, C, DE1, and F deletion fragments) spanning the genome are flanked by unique interconnecting BglI and BstXI sites, allowing for directional assembly into a full-length replicon cDNA by in vitro ligation. TGEV A contained a unique T7 start site at the 5' end and the deletion fragments (FiGFP2-AvrII and FiGFP2-EcoNI) contain GFP and a 25-nt T tail, allowing for the synthesis of capped T7, polyadenylated transcripts in vitro.

FIG. 2 depicts the growth kinetics of recombinant TGEV. ST cells were infected with TGEV GFP recombinant viruses or icTGEV 1000.

FIG. 3A shows RT-PCR products of leader-containing GFP transcripts from cells transfected with TGEV-Rep(AvrII). Arrow indicates leader-containing GFP amplicon.

FIG. 3B shows RT-PCR products of GFP derived from cells transfected with TGEV-Rep(EcoNI). Arrow indicates leader-containing GFP amplicon.

FIG. 3C shows the sequence of the leader-containing GFP transcripts which originate from the 3A TSE site. In this context, the 21-nucleotide N TSE site is not recognized as an initiator of subgenomic transcription.

FIG. 4A depicts the structure of constructs expressing recombinant PRRS GP5.

FIG. 4B shows the icTGEV PRRS GP5 leader-containing transcripts in infected cells. A 1-kb ladder is shown in lane 1.

FIG. 5 depicts the structure of the TGEV N gene rearranged viruses. The TGEV N gene and TSE were inserted downstream of the 3A TSE site by removing the ORF 3A (TGEV 2N(PflMI)) or ORF 3A and a part of ORF 3B (TGEV 2N(ScaI)). The downstream N gene was flanked by restriction sites and removed to produce the SNEM rearranged viruses.

FIG. 6 shows recombinant virus growth. Viruses were inoculated into ST cells. Note that the TGEV SNEMp4A isolate was not robust and was isolated at passage 4 after the initial transfection of full-length SNEM transcripts into cells. In contrast, TGEV SNEM-1 and SNEM-4 viruses were plaque purified from passage 9 following transfection. TGEV SNEM1 p15A and SNEM4 p15A were isolated following 15 serial passages of TGEV SNEM 1 and 4 viruses in ST cells, respectively, and despite N rearrangement replicate to titers approaching that of wild-type virus.

FIG. 7 shows the sequence of the RT-PCR leader-containing N transcripts (TXPT). TGEV SNEM1 leader containing N transcripts were sequenced. Five of seven N transcripts initiated from the 3A TSE site and two initiated from the N TSE site.

FIG. 8 graphically depicts the genetic organization of TGEV SNEM 1 and 4. The TGEV F domain was cloned from TGEV SNEM1 and SNEM4 infected cells. The major alteration was a deletion of residual ORF 3B sequences in the original SNEM Clone F sequence. Note that the E TSE site at position 25,813 is deleted in the TGEV SNEM1 and 4 viruses.

FIG. 9 depicts the strategy to assemble TGEV-Rep(AvrII) VRPs. In the full-length TGEV-Rep(AvrII) cDNA construct, ORF 3A has been replaced with GFP, and ORF 3B and the 5' end of the E gene have been deleted. To produce packaged replicon particles, replicon RNA-transfected cells were infected with VEE VRPs expressing the TGEV E protein [VEE-TGEV(E)]. Alternatively, TGEV-Rep(AvrII) replicon RNAs can be co-electroporated with pVR21-E1-derived transcripts. TGEV VRPs should be released from cells that can be used as single-hit expression vectors.

FIG. 10 shows the growth kinetics of wild-type (WT) TGEV alone and TGEV with VEE VRPs expressing a G.sub.1 Norwalk-like virus capsid (WT+VEE).

FIG. 11A shows RT-PCR products of GFP transcripts derived from cells transfected with TGEV VRPs (lane 1). A 1-kb ladder is shown in lane 2. Arrow indicates leader-containing GFP amplicon.

FIG. 11B shows RT-PCR products of N and M subgenomic transcripts derived from cells transfected with TGEV VRPs. A 1-kb ladder is shown in lane 1. Arrows indicate leader-containing M (lane 2) or N (lane 3) amplicons.

FIG. 12 shows the growth kinetics of TGEV replicon. Plaque assays were performed on TGEV-GFP2 (closed diamond) and TGEV VRP (closed square)-infected cultures.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

"Nidovirus" as used herein refers to viruses within the order Nidovirales, including the families Coronaviridae and Arteriviridae. All viruses within the order Nidovirales share the unique feature of synthesizing a nested set of multiple subgenomic mRNAs. See M. Lai and K. Holmes, Coronaviridae: The Viruses and Their Replication, in Fields Virology, pg 1163, (4.sup.th Ed. 2001). Particular examples of Coronaviridae include, but are not limited to, toroviruses and coronaviruses.

"Coronavirus" as used herein refers to a genus in the family Coronaviridae, which family is in turn classified within the order Nidovirales. The coronaviruses are large, enveloped, positive-stranded RNA viruses. They have the largest genomes of all RNA viruses and replicate by a unique mechanism which results in a high frequency of recombination. The coronaviruses include antigenic groups I, II, and III. While the present invention is described primarily with respect to porcine transmissible gastroenteritis virus (TGEV), the invention may be carried out with any coronavirus, such as human respiratory coronavirus, porcine respiratory coronavirus, canine coronavirus, feline enteric coronavirus, feline infectious peritonitis virus, rabbit coronavirus, murine hepatitis virus, sialodacryoadenitis virus, porcine hemagglutinating encephalomyelitis virus, bovine coronavirus, avian infectious bronchitis virus, and turkey coronavirus. See generally M. Lai and K. Holmes, Coronaviridae: The Viruses and Their Replication, in Fields Virology, (4.sup.th Ed. 2001).

A "nidovirus permissive cell" as used herein can be any cell in which a coronavirus can at least replicate, including both naturally occurring and recombinant cells. In some embodiments the nidovirus permissive cell is also one which the nidovirus can infect. The nidovirus permissive cell may be one which has been modified by recombinant means to express a cell surface receptor for the nidovirus

A "replicon RNA" as used herein refers to RNA that is packaged into coronavirus particles within a helper cell. The replicon RNA may be introduced into the helper cell by any suitable means, including but not limited to electroporation of the RNA, transient or stable transfection of the helper cell with a DNA that transcribes the replicon RNA, etc.

A "helper RNA" as used herein refers to an RNA that encodes a structural protein absent from a corresponding replicon RNA. The helper RNA may be introduced into the helper cell by any suitable means, including but not limited to electroporation of the RNA, transient or stable transfection of the helper cell with a DNA that transcribes the helper RNA, etc.

A "heterologous RNA" as described herein may encode anything which it is desired to administer into a subject for any purpose. For example, the heterologous RNA may encode, and express in the subject, a protein or peptide. The protein or peptide may, for example, be an antigen or immunogen, for example where it is desired to raise antibodies in an animal subject, which antibodies can be collected and used for diagnostic or therapeutic purposes, or where it is desired to elicit an immune response to the protein or peptide in subject for producing at least a partial protective immune response to the protein or peptide in the subject.

A "structural protein" as used herein refers to a protein required for production of viral particles, such as those encoded by the S, E, M and N genes. Where replicon or helper RNAs lack sequences encoding the structural protein, the sequences may be wholly or partly deleted, so long as the sequence is effectively incapable of producing the necessary protein in functional form.

"Replication defective" as used herein means that the viral genome contained within viral particles produced by the present invention cannot of themselves produce new viral particles.

Subjects which may be administered or treated by the viral particles or VLPs of the present invention may be any subject, generally vertebrates, for which the viral particles or VLPs are infectious, including but not limited to birds and mammals such as pigs, mice, cows, and humans).

While the invention is sometimes described with particular reference to coronaviruses and TGEV below, it will be understood that this teaching is applicable to other nidoviruses and its families (as described above) as well.

The present invention may be implemented in any of a varieties of ways, including by techniques, compositions and formulations known in the art (see, e.g., U.S. Pat. No. 6,156,558 to Johnston et al.; U.S. Pat. No. 5,639,650 to Johnston et al.) modified in light of the teaching set forth above and below. Applicants specifically intend that the disclosures of all United States patent references cited herein be incorporated herein by reference in their entirety.

The present invention describes the assembly of recombinant transmissible virus and replicons that express heterologous genes which can be used to make vaccines against homologous and heterologous pathogens (Agapov, et al. (1998) Proc. Natl. Acad. Sci. USA 95:12989-12994; Balasuriya, et al. (2000) J. Virol. 74:10623-10630; Berglund, et al. (1998) Nat. Biotechnol. 16:562-565; Bredenbeek, et al. (1993) J. Virol. 67:6439-6446; DiCiommo and Bremner (1998) J. Biol. Chem. 273:18060-18066; Dollenmaier, et al. (2001) Virology 281:216-230; Dubensky, et al. (1996) J. Virol. 70:508-519; Hevey, et al. (1998) Virology 251:28-37; Johanning, et al. (1995) Nucleic Acids Res. 23:1495-1501; Khromykh (2000) Curr. Opin. Mol. Ther. 2:555-569; Khromykh and Westaway (1997) J. Virol. 71:1497-1505; Liljestrom and Garoff (1991) Bio/Technology 9:1356-1361; Percy, et al. (1992) J. Virol. 66:5040-5046; Porter, et al. (1993) J. Virol. 67:3712-3719; Pushko, et al. (2000) Vaccine 19:142-153; Schultz-Cherry, et al (2000) Virology 278:55-59; Varnavski and Khromykh (1999) Virology 255:366-375; Varnavski, et al. (2000) J. Virol. 74:4394-4403).

The use of replicons as a vaccine delivery system offers a number of important advantages over the use of live, attenuated virus vaccines, which are capable of independent spread and recombination with wild-type virus populations. Replicon vectors are an inherently safer alternative to the use of live, attenuated virus vaccines due to the lack of progeny virus production. In addition, high-level expression of heterologous genes can result in the use of a relatively low dose of virus replication particles (VRPs) for vaccination and inmmune induction. Moreover, gene order rearranged viruses will be inherently more stable and less pathogenic than attenuated wild-type strains.

Coronavirus vectors such as TGEV vectors provide a system for the incorporation and expression of one or more foreign genes, as coronaviruses contain a polycistronic genome organization and synthesize multiple subgenomic-length mRNAs (Enjuanes and van der Zeijst (1995) In: S. G. Siddell (ed.), The Coronaviridae. Plenum Press, New York, N.Y., p. 337-376). It has been shown that TGEV ORFs 3A and 3B likely encode luxury functions that can be deleted without affecting infectivity or replication in vitro and may serve as appropriate sites for the insertion of heterologous genes into the genome (Enjuanes and van der Zeijst (1995) In:

S. G. Siddell (ed.), The Coronaviridae. Plenum Press, New York, N.Y., p. 337-376; Laude, et al. (1990) Vet. Microbiol. 23:147-154; McGoldrick, et al. (1999) Arch. Virol. 144:763-770; Vaughn, et al. (1995) J. Virol. 69:3176-3184; Wesley, et al. (1991) J. Virol. 65:3369-3373). In contrast to arteriviruses, the coronavirus transcription start elements (TSEs) rarely overlap, or overlap slightly, with upstream ORFs, simplifying the design and expression of foreign genes from downstream TSEs (Chen, et al. (1995) Virus Res. 38:83-89; Eleouet, et al. (1995) Virology 206:817-822; Tung, et al. (1992) Virology 186:676-683). The helical nucleocapsid structure of coronaviruses such as TGEV also minimize packaging constraints and allow for the expression of multiple large genes from a single construct (Enjuanes and van der Zeijst (1995) In: S. G. Siddell (ed.), The Coronaviridae. Plenum Press, New York, N.Y., p. 337-376; Lai and Cavanagh (1997) Adv. Virus Res. 48:1-100; Risco, et al. (1996) J. Virol. 70:4773-4777). Importantly, recombinant coronavirus VRPs can be readily targeted to other mucosal surfaces mammalian species such as swine by simple replacements in the S glycoprotein gene, which has been shown to determine tissue and species tropism (Delmas, et al. (1992) Nature 357:417-420; Kuo, et al. (2000) J. Virol. 74:1393-1406; Sanchez, et al. (1999) J. Virol. 73:7607-7618; Tresnan, et al. (1996) J. Virol. 70:8669-8674). For these reasons, coronavirus VRPs and vectorsprovide a valuable approach for the production of combination vaccines in a variety of mammalian hosts.

Accordingly, one aspect the present invention relates to a method for inducing an antigenic and/or immunological response in a vertebrate to a pathogen by inoculating the vertebrate with a recombinant coronavirus virus modified by the presence, in a nonessential region of the coronavirus genome, of nucleic acid from any source which encodes for and expresses an antigen of the pathogen, or a protein or peptide for which an antibody is desired.

In a further aspect, the present invention is directed to a method for expressing a gene product or inducing an antigenic or immunological response to an antigen in a vertebrate with a recombinant virus which does not productively replicate in the cells of the vertebrate but which does express the gene product or the antigen in those cells.

The methods can comprise inoculating the vertebrate with the recombinant virus, e.g., by introducing the virus into the vertebrate subcutaneously, intradermally, intramuscularly, orally or in ovum.

The antigen or antigenic protein or peptide encoded by the heterologous RNA and expressed in the host can be an antigen of a vertebrate pathogen, e.g., a mammalian pathogen or a swine pathogen, such as a rabies G antigen, gp51, 30 envelope antigen of bovine leukemia virus, FeLV envelope antigen of feline leukemia virus, glycoprotein D antigen of herpes simplex virus, a fusion protein antigen of the Newcastle disease virus, an RAV-1 envelope antigen of rous associated virus, nucleoprotein antigen of avian or mammalian influenza virus, a fusion protein antigen of porcine reproductive and respiratory disease virus (PRRSV), a matrix antigen of the infectious bronchitis virus, a glycoprotein species of PRRSV or a peplomer antigen of the infectious brochitis virus.

In another aspect, the present invention is directed to synthetic recombinant coronavirus modified by the insertion therein of DNA or RNA from any source, and particularly from a non-coronavirus or non-TGEV source, into a nonessential region of the TGEV genome. Synthetically modified TGEV virus recombinants carrying exogenous (i.e. non-coronavirus) nucleic acids or genes encoding for and expressing an antigen, which recombinants elicit the production by a vertebrate host of immunological responses to the antigen, and therefore to the exogenous pathogen, are used according to the invention to create novel vaccines which avoid the drawbacks of conventional vaccines employing killed or attenuated live organisms, particularly when used to inoculate vertebrates.

The present invention demonstrates that the Green Fluorescent Protein (GFP) and the PRRSV surface glycoprotein (GP5) can be inserted and expressed from the TGEV genome, demonstrating the feasibility of using TGEV-based replicon vectors for heterologous gene expression. Efficient self-replication of in vitro-transcribed recombinant TGEV and replicon RNAs was demonstrated by GFP expression, the presence of leader-containing subgenomic transcripts, and the production of infectious recombinant virus and VRPs. These data support previous results suggesting that the TGEV ORFs 3A and 3B were not required for virus replication

in vitro, although these genes may confer subtle fitness advantages that cannot be detected by these assays (Laude, et al. (1990) Vet. Microbiol. 23:147-154; McGoldrick, et al. (1999) Arch. Virol. 144:763-770; Wesley, et al. (1991) J. Virol. 65:3369-3373). It should also be possible to determine the minimal TGEV replicon size by serially deleting each of the downstream ORFs. However, this may be complicated by the requirement of RNA secondary structures that may be essential for genome replication or subgenomic RNA synthesis (Hsue, et al. (2000) J. Virol. 74:6911-6921; Hsue, et al. (1997) J. Virol. 71:7567-7578; Williams, et al. (1999) J. Virol. 73:8349-8355). As has been previously reported, efficient TGEV RNA transfection and expression in cells were significantly enhanced upon the co-electroporation of N transcripts (Wesley, et al. (1991) J. Virol. 65:8349-8355). Because the nucleocapsid protein interacts with leader and negative-strand RNA and colocalizes with the viral polymerase sites of RNA synthesis, it is possible that the N protein may function as part of the transcription complex in some undetermined manner (Baric, et al. (1988) J. Virol. 62:4280-4287; Denison, et al. (1999) J. Virol. 73:6862-6871). Replicon constructs containing N gene deletions may facilitate studies of the possible N gene function(s) in TGEV replication. Using synthetic defective-interfering (DI) RNA genomes, some groups have reported that downstream TSEs suppress transcription from upstream TSEs (Joo and Makino (1995) J. Virol. 69:272-280; Krishnan, et al. (1996) Virology 218:400-405). In addition, data suggest that the N gene TSE is the strongest initiator of TGEV subgenomic RNA transcription (Hiscox, et al. (1995) Virus Res. 36:119-130; Jacobs, et al. (1986) J. Virol. 57:1010-1015). In the recombinant virus and replicon RNAs described herein, GFP subgenomic mRNA synthesis was initiated from the normal ORF 3A TSE rather than from the 20-nt N gene TSE that has been duplicated just upstream of gfp (FIG. 1B). The results presented herein do not necessarily contradict earlier reports, as in this context the N gene TSE function was silent and would not display the reported phenotypes. Also, fundamental differences exist between the two systems used in these analyses (DI versus nearly full-length replicon), including the rapid replication of small .about.2- to 3-kb DI RNAs and TSE presentation, compared with 28.5-kb genome-length RNAs. For example, experiments utilizing DI systems involved TSE elements within primary and secondary flanking genome sequence contexts that were not authentic, while the TSEs in the recombinant viruses and replicons presented herein closely approximate the wild-type TGEV genome. These data suggest that TSE location and flanking sequences likely have an impact on gene expression, especially in promoter proximal locations, which are fundamentally different in the two systems. The simplest interpretation of these data is that the random insertion of a large .about.20-nt TSE element is not sufficient to initiate TGEV subgenomic mRNA synthesis unless this TSE is provided to the viral transcriptional machinery in an appropriate context that as of yet remains unknown. These data, as well as that obtained concerning bovine coronavirus (Pancholi, et al. (2000) J. Infect. Dis. 182:18-27), has lead to the hypothesis that TGEV subgenomic RNA transcription may be mediated by long-range RNA and/or ribonucleoprotein interactions, which are most definitely dependent on higher orders of genome structure.

Smaller leader-containing RNAs were noted in the present invention, indicating the presence of cryptic transcription start sites within GFP. This phenomenon was previously observed following expression of GFP from the MHV genome (Fischer, et al. (1997) J. Virol. 71:5148-5160; Schaad and Baric (1993) Virology 196:190-198). Both the TGEV and MHV genomes contain a number of atypical start sites that result in the transcription of aberrant subgenomic RNAs (Fischer, et al. (1997) J. Virol. 71:5148-5160). These data further substantiate the conclusion that the insertion of TSE elements may not be enough to direct TGEV subgenomic mRNA synthesis and that genome location, flanking sequence, and secondary sequence likely function in this process.

The SNEM viruses and their revertants provided in the present invention will also provide new opportunities for the development of safe and effective coronavirus vaccines and heterologous expression vectors. Gene order mutants and their revertant viruses will likely be attenuated in animals, but allow for high level expression of foreign genes (Wertz, et al. (1998) Proc. Natl. Acad. Sci. USA 95:3501-3506). It is believed that recombination with wild-type strains may result in attenuated progeny, either from the gene order rearrangement, or from the compensatory mutations, which will likely enhance TGEV SNEM virus replication, but prove detrimental to wild-type TGEV virus fitness and replication.

The VEE, Kunjin, and Sindbis replicon packaging systems involve the co-transfection of replicon and helper RNAs that express the structural genes (Pushko, et al. (1997) Virology 239:389-401). In addition to this method, replicon RNAs have been packaged by the expression of structural genes in trans from wild-type or mutated

virus (Nugent, et al. (1999) J. Virol. 73:427-435; Percy, et al. (1992) J. Virol. 66:5040-5046). An important concern with these types of replicon systems is the production of recombinant virus, especially when considering the development of a replicon particle vaccine. Recombinant viruses have been isolated from Sindbis virus and VEE virus replicon systems that may be the result of recombination between replicon and helper RNAs and/or co-packaging of replicon and helper RNAs into the same VRP (Frolov, et al. (1997) J. Virol. 71:2819-2829; Geigenmuller-Gnirke, et al. (1991) Proc. Natl. Acad. Sci. USA 88:3253-3257; Pushko, et al. (1997) Virology 239:389-401). Coronaviruses undergo homologous recombination at high frequencies during mixed infection, which presents a significant concern for the production and use of TGEV replicons (Baric, et al. (1990) Virology 177:646-656; Fu and Baric (1992) Virology 189:88-102; Mendez, et al. (1996) Virology 217:495-507). The coronavirus replicon packaging system of the present invention involves, in one embodiment, the use of an unrelated virus vector (VEE). In one embodiment of this system, coronavirus-Rep(AvrII) RNAs were packaged by the expression of TGEV E in trans from VEE VRPs as well as from pVR21-E1-derived helper RNA transcripts. Because the TGEV replicon packaging system involves the use of an unrelated virus vector (VEE), the possibility of a recombination event between replicon and helper RNAs may be reduced. In addition, the TGEV E gene carried in the VEE replicon construct lacks an appropriate TSE, which was necessary for the initiation of subgenomic RNA transcription. As this same TSE was deleted in the TGEV-Rep(AvrII) construct, expression of TGEV helper genes that are recombined into TGEV replicon RNAs should be minimized in this system. Although recombinant wild-type TGEV was not detected, this concern could also be reduced by the inclusion of attenuating mutations in the helper proteins and/or the engineering of a bipartite replicon and helper system (TGEV E and M structural proteins) (Pushko, et al. (1997) Virology 239:389-401). Nevertheless, the possibility of RNA recombination between the replicon RNA and wild-type virus cannot be eliminated and remains an important issue requiring additional analysis.

VEE replicon vectors may be used to express coronavirus structural genes in producing combination vaccines. Dendritic cells, which are professional antigen-presenting cells and potent inducers of T-cell responses to viral antigens, are preferred targets of VEE and VRP infection, while TGEV targets the mucosal surfaces of the respiratory and gastrointestinal tract (Banchereau and Steinman (1998) Nature 392:245-252; Enjuanes, et al. (1995) Dev. Biol. Stand. 84:145-152; Enjuanes and van der Zeijst (1995) In: S. G. Siddell (ed.), The Coronaviridae. Plenum Press, New York, N.Y., p. 337-376; MacDonald and Johnston (2000) J. Virol. 74:914-922). As the VEE and TGEV replicon RNAs synergistically interact, two-vector vaccine systems are feasible that may result in increased immunogenicity when compared with either vector alone. Combination prime-boost vaccines (e.g., DNA immunization and vaccinia virus vectors) have dramatically enhanced the immune response (notably cellular responses) against target papillomavirus and lentivirus antigens compared to single-immunization regimens (Chen, et al. (2000) Vaccine 18:2015-2022; Gonzalo, et al. (1999) Vaccine 17:887-892; Hanke, et al. (1998) Vaccine 16:439-445; Pancholi, et al. (2000) J. Infect. Dis. 182:18-27). Using different recombinant viral vectors (influenza and vaccinia) to prime and boost may also synergistically enhance the immune response, sometimes by an order of magnitude or more (Gonzalo, et al. (1999) Vaccine 17:887-892). The strategy presented herein for the assembly of TGEV replicon constructs was based on the use of six cDNA subclones that span the entire length of the TGEV genome, designated fragments A, B 1, B2, C, D/E, and F (Yount, et al. (2000) J. Virol. 74:10600-10611). Each fragment is flanked by restriction sites that leave unique interconnecting junctions of 3 or 4 nt in length (BglI and BstXI). These sticky ends are not complementary to most other sticky ends generated with the same enzyme at other sites in the DNA, allowing for the systematic assembly of TGEV cDNAs by in vitro ligation.

This strategy is capable of circumventing problems associated with genome size constraints as well as regions of chromosomal instability, while allowing for simple reverse genetic applications. In this application, the full-length TGEV cDNA constructs must be synthesized de novo and do not exist intact in bacterial vectors, circumventing problems with sequence instability. However, this did not restrict the applicability of this approach. In fact, the separation of the replicon constructs into distinct fragments allows for genetic manipulation of independent subclones, thereby minimizing the occurrence of spurious mutations that arise during recombinant DNA manipulation. Because replicon cDNAs are consumed during in vitro transcription in this strategy, a weakness of this approach is that the full-length replicon cDNAs are consumed with use and must be continually rebuilt. However, replicon cDNAs will likely be stable in bacterial artificial chromosome vectors after reverse genetic manipulations, preventing the need for repetitive de novo synthesis by engineered

constructs (Almazan, et al. (2000) Proc. Natl. Acad. Sci. USA 97:5516-5521; Messerle, et al. (1997) Proc. Natl. Acad. Sci. USA 94:14759-14763).

The synthesis of large RNA transcripts (.about.27 to 29 kb) in vitro is problematic, and the electroporation of such large RNA constructs, even in the presence of enhancing N transcripts, has also proven difficult, resulting in a 1% transfection efficiency. Therefore, transfecting cells with helper packaging constructs and subsequently passing the coronavirus VRPs in the presence of VEE-TGEV(E) VRPs can address this issue. In this way, VRPs can be amplified and high concentrations may amplify replicon titers for future applications. In addition, the use of a DNA launch platform, such as with a cytomegalovirus promoter, may be used to overcome any problems associated with an RNA launch system.

Helper or replicon RNAs (and their corresponding DNAs) that contain two or more genes optionally but preferably include a gene order rearrangement to atttenuate (e.g., reduce the virulence) as compared to a corresponding wild-type virus that did not contain such a gene order rearrangment (i.e., comparing a virus with all of the genes and the order rearrangement with a wild-type virus). Depending upon the number of genes within the helper or replicon RNA, it may contain two, three, or four or more gene order rearrangements. The wild-type order is reflected in the first transcript shown in FIG. 1A, and is, from 5' to 3', the gene order of: S, 3A, 3B, E, M, N, and HP. For example, modified orders for the replicon RNA, when the helper RNA contains the E gene, may include: S, 3B, M, N, HP and 3A; 3A, 3B, M, N, HP and S; S, 3A, 3B, N, Hp, and M; etc. In other embodiments, the E gene may be provided alone on the replicon RNA, and the helper RNA may contain the genes described above in the orders given above. Modified orders for the replicon RNA, when the helper RNA contains the M and N (in natural or reverse order) genes, may include: 3A, 3B, E, S and HP; S, E, 3A, 3B, and HP; S, 3A, 3B, E, and HP; S, E, HP, 3A, and 3B, etc. In other embodiments, the replicon RNA may contain the M and N genes (in natural or reverse orders), and the remaining genes may be provided on the helper RNA in orders such as given above. In still other embodiments, where the helper RNA contains the S gene, the replicon RNA may contain the remaining genes in the order 3B 3A, E, M, N, and HP; 3A, 3B, E, N, M, and HP; 3B, 3A, E, N, M, and HP; etc. Again, the replicon RNA may contain the S gene, and the helper RNA may contain the remaining genes in the orders given above. The 3A, 3B, and HP genes are nonessential and some or all may be deleted, or they may be included in an alternate order to serve as attenuating mutations. The genes may be divided among multiple helper RNAs, some or all of which contain gene order rearrangements. The foregoing examples are merely illustrative, and numerous additional variations will be readily apparent to those skilled in the art.

In other embodiments, an attenuating mutation can be introduced by deleting one or more of the nonessential genes 3A, 3B, and HP.

The infectious, replication defective, nidovirus particles may be prepared according to the methods disclosed herein in combination with techniques known to those skilled in the art. The method includes transfecting an nidovirus-permissive cell with a replication defective replicon RNA including the nidovirus packaging segment and an inserted heterologous RNA, a first helper RNA including RNA encoding at least one nidovirus structural protein, and a second helper RNA including RNA encoding at least one nidovirus structural protein which is different from that encoded by the first helper RNA; producing the nidovirus particles in the transfected cell; and collecting the nidovirus particles from the cell. The step of transfecting the nidovirus-permissive cell can be carried out according to any suitable means known to those skilled in the art. For example, uptake of the RNA into the cells can be achieved by any suitable means, such as for example, by treating the cells with DEAE-dextran, treating the cells with "LIPOFECTIN.TM.", and by electroporation, with electroporation being the currently preferred means of achieving RNA uptake into the nidovirus-permissive cells. These techniques are well known in the art. See e.g., U.S. Pat. No. 5,185,440 to Davis et al., and PCT Publication No. WO 92/10578 to Bioption A B, the disclosures of which are incorporated herein by reference in their entirety.

The step of producing the infectious viral particles in the cells may also be carried out using conventional techniques. See e.g., U.S. Pat. No. 5,185,440 to Davis et al., PCT Publication No. WO 92/10578 to Bioption A B, and U.S. Pat. No. 4,650,764 to Temin et al. (although Temin et al., relates to retroviruses rather than nidoviruses). The infectious viral particles may be produced by standard cell culture growth techniques.

The step of collecting the infectious nidovirus particles may also be carried out using conventional techniques. For example, the infectious particles may be collected by cell lysis, or collection of the supernatant of the cell culture, as is known in the art. See e.g., U.S. Pat. No. 5,185,440 to Davis et al., PCT Publication No. WO 92/10578 to Bioption A B, and U.S. Pat. No. 4,650,764 to Temin et al. (although Temin et al. relates to retroviruses rather than nidoviruses). Other suitable techniques will be known to those skilled in the art. Optionally, the collected infectious nidovirus particles may be purified if desired. Suitable purification techniques are well known to those skilled in the art.

Pharmaceutical formulations, such as vaccines or compositions for producing antibodies in animals of the present invention comprise an antigenic or immunogenic amount of the infectious, replication defective nidovirus particles as disclosed herein in combination with a pharmaceutically acceptable carrier. An "antigenic amount" is an amount of the nidovirus particles which is sufficient to evoke the production of antibodies to the encoded antigenic protein or peptide contained and expressed on the heterologous RNA. An "immunogenic amount" is an amount of the infectious nidovirus particles which is sufficient to evoke an immune response in the subject to which the pharmaceutical formulation is administered. An amount of from about 10.sup.3, 10.sup.5 or 10.sup.7 to about 10.sup.10 or 10.sup.12 plaque forming units per dose is believed suitable, depending upon the age and species of the subject being treated, and the purpose of the treatment. Exemplary pharmaceutically acceptable carriers include, but are not limited to, sterile pyrogen-free water and sterile pyrogen-free physiological saline solution. Subjects which may be administered immunogenic amounts of the infectious, replication defective nidovirus particles of the present invention include but are not limited to human and animal (e.g., horse, donkey, mouse, hamster, monkeys) subjects. Administration may be by any suitable means, such as intraveneous, intraperitoneal or intramuscular injection.

The examples, which follow, are set forth to illustrate the present invention, and are not to be construed as limiting thereof. In the following examples, mM means millimolar, .mu.g means microgram, ml means milliliter, .mu.l means microliter, V means volt, .mu.F means microfarad, cm means centimeter, h means hour, ORF means open reading frame, GFP means green fluorescent protein, PBS means phosphate-buffered saline, M means molar, s means second, nt means nucleotide, and min means minute.

EXAMPLE 1

Virus and Cells

The Purdue strain of TGEV (ATCC VR-763) was obtained from the American Type Culture Collection and passaged once in swine testicular (ST) cells. ST cells were obtained from the American Type Culture Collection (ATCC 1746-CRL) and maintained in minimal essential medium containing 10% fetal clone II (HyClone Laboratories, Inc., Logan, Utah) and supplemented with 0.5% lactalbumin hydrolysate, 1.times. nonessential amino acids, 1 mM sodium pyruvate, kanamycin (0.25 .mu.g/ml), and gentamicin (0.05 .mu.g/ml). Baby hamster kidney (BHK) cells (BHK-21 [ATCC CCL10]) were maintained in alpha-minimal essential medium containing 10% fetal calf serum supplemented with 10% tryptose phosphate broth, kanamycin (0.25 .mu.g/ml), and gentamicin (0.05 .mu.g/ml). To determine the effect of co-infection with TGEV and VEE VRPs on TGEV growth rate, cultures of ST cells (5.times.10.sup.5) were infected with wild-type TGEV alone or with wild-type TGEV and VEE VRPs encoding a Norwalk-like virus (VEE-NCFL) capsid antigen (ORF 2) at a multiplicity of infection (MOI) of 5 for 1 h (Harrington, et al. (2002) J. Virol. 76:730-742). The cells were washed twice with phosphate-buffered saline (PBS) to remove residual virus and VEE VRPs, and the cells were subsequently incubated at 37.degree. C. in complete medium. At different times post-infection, progeny virions were harvested and assayed by plaque assay in ST cells, as previously described (Yount, et al. (2000) J. Virol. 74:10600-10611).

EXAMPLE 2

Recombinant DNA Manipulations of TGEV F Subclone

Plasmid DNAs were amplified in Escherichia coli DH5.alpha. and purified with the QIAprep Miniprep kit (Qiagen Inc., Valencia, Calif.). All enzymes were purchased from New England BioLabs (Beverly, Mass.) and used according to the manufacturer's directions. DNA fragments were isolated from Tris-acetate-EDTA agarose gels (0.8%) with the QIAEX II gel extraction kit (Qiagen Inc.). All DNA was visualized using Dark Reader technology (Clare Chemical Research, Denver, Colo.) to prevent UV-induced DNA damage that could impact subsequent manipulations, including in vitro transcription. It was found that increased concentrations of full-length transcripts and increased transfection efficiencies were achieved after Dark Reader technology was used to isolate the TGEV cDNAs.

Six subgenomic cDNA clones (A to F) spanning the entire TGEV genome were isolated using standard molecular techniques as previously described (Yount, et al. (2000) J. Virol. 74:10600-10611). The 3' end of the TGEV genome, carrying the S, ORF 3A, ORF 3B, E, M, N, and ORF S genes, is contained within the 5.1-kb TGEV F subclone. To generate TGEV cDNA constructs containing a reporter gene, nucleotides 24828 to 25073 (GenBank accession no. AJ271965), corresponding to ORF 3A, were removed and replaced with the ClaI and PflMI restriction sites using conventional recombinant DNA techniques such that the adjacent ORFs (S and 3B) were not disrupted (Sambrook, et al. (1989) Molecular Cloning: a Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). The ClaI site was inserted 25 nt downstream of the 3A TSE, while the PflMI and ScaI sites are located just upstream of the ORF 3B TSE or downstream of the ORF 3B ATG start codon, respectively (FIG. 1A). To potentially enhance GFP expression, the mammalian codon-optimized version of the GFP gene was isolated from the noncytopathic Sindbis virus vector pSINrep19/GFP (kindly provided by Charlie Rice, Columbia University) and was inserted with or without a 5' 20-nt N gene TSE (TGGTATAACTAAACTTCTAA; SEQ ID NO:17) into the TGEV genome (FIG. 1B). The TGEV ORF 3A (ClaI/PflMI digestion), and in some instances a portion of ORF 3B (ClaI/ScaI digestion), were removed and replaced with GFP in several orientations (FIG. 1A) using standard recombinant DNA techniques (Sambrook, et al. (1989) Molecular Cloning: a Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). In TGEV iGFP2(ScaI), an ATG start codon was inserted between the ORF 3A and N TSEs to ablate expression from ORF 3A TSE-derived transcripts (FIG. 1B). Clones were identified by DNA sequencing using an ABI model 377 automated sequencer and constructs TGEV pFiGFP2(PflMI) and TGEV iGFP2(ScaI) were subsequently used in the assembly of recombinant TGEV viral cDNA and as the backbone for the construction of structural gene deletions (FIG. 1D).

EXAMPLE 3

Assembly of Full-Length TGEV cDNAs

The six cDNA subclones spanning the entire TGEV genome, including the FiGFP2(PflMI) and FiGFP2(PflMI) deletion subclones, were used to assemble TGEV viral and replicon constructs, respectively, as previously described (FIG. 1D) (Yount, et al. (2000) J. Virol. 74:10600-10611; Curtis, et al. (2002) J. Virol. 76:1422-1434). The TGEV A fragment contains a T7 promoter while the TGEV FiGFP2(PflMI), FiGFP2-AvrII, and FiGFP2-EcoNI fragments terminate in a 25-nt poly(T) tract and a unique NotI site at the 3' end, allowing for in vitro T7 transcription of capped, polyadenylated transcripts (FIG. 1C). To assemble full-length TGEV recombinant virus and subgenomic replicon cDNAs, plasmids were digested with BglI and BstXI or NotI, and the appropriately-sized inserts were isolated from agarose gels. The TGEV A-B1, B2-C, and DE-1-FiGFP2 fragments were ligated overnight at 4.degree. C. in the presence of T4 DNA ligase, according to the manufacturer's directions. Systematically, assembled products were isolated and extracted from agarose gels, and the TGEV A-B1, B2-C, and DE-1-FiGFP2 fragments were religated overnight. The final ligation products were purified by phenol-chloroform-isoamyl alcohol and chloroform extraction, precipitated under isopropanol, and washed with 70 and 90% ethanol. Purified TGEV full-length viral and replicon cDNA constructs, designated TGEV-GFP2(PflMI), TGEV-Rep(AvrII), and TGEV-Rep(EcoNI), were subsequently used for T7 in vitro transcription. The resulting replicon RNAs from Rep(AvrII) and TGEV-Rep(EcoNI) T7 in vitro transcription were .about.29.1 kb and 28.4 kb, respectively.

EXAMPLE 4

TGEV in vitro Transcription and Transfection

The TGEV A fragment contains a T7 promoter while the TGEV FiGFP2(PflMI) fragment has a poly(T) tract at its very 3' end, allowing for in vitro T7 transcription of capped, polyadenylated mRNAs. Capped, runoff T7 transcripts were synthesized in vitro from assembled TGEV and replicon cDNAs using the MMESSAGE MMACHINE.TM. kit as described by the manufacturer (Ambion, Austin, Tex.), with certain modifications. TGEV RNA transcription reaction mixtures (50-.mu.l volume) were prepared containing 7.5 .mu.l of a 30 mM GTP stock and incubated at 37.degree. C. for 2 h. Similar reactions were performed using 1 .mu.l of PCR amplicons carrying the TGEV N gene sequence and 1 .mu.g of pVR21-E1, each containing a 2:1 ratio of cap analog to GTP. A portion of the RNA transcripts (5 .mu.l of the 50-.mu.l reaction volume) were treated with DNase I, denatured, and separated in 0.5% agarose gels in Tris-acetate-EDTA buffer containing 0.1% sodium dodecyl sulfate. The remaining RNA transcripts were mixed with transcripts encoding TGEV N and directly electroporated into BHK cells. As a control, separate transcription reaction mixtures were treated with RNase A for 15 min at room temperature prior to transfection. Using transcripts driven from various pSin replicons as a control, it was predicted that the transcripts generated from the replicon cDNAs were likely of the appropriate lengths.

BHK cells were grown to subconfluence (.about.70%), trypsinized, washed twice with PBS, and resuspended in PBS at a concentration of 10.sup.7 cells/ml. RNA transcripts were added to 800 .mu.l of the cell suspension (8.times.10.sup.6 cells) in an electroporation cuvette, and three electrical pulses of 850 V at 25 .mu.F were given with a Bio-Rad Gene Pulser II electroporator. N gene transcripts (lacking the TGEV leader sequence) were included in all electroporations, as these transcripts may enhance the recovery of infectious TGEV virions derived from the full-length cDNA construct, TGEV 1000 (Yount, et al. (2000) J. Virol. 74:10600-10611). The BHK cells were either seeded alone or, in some instances, mixed with 10.sup.6 ST cells in a 75-cm.sup.2 flask and incubated at 37.degree. C. in 5% CO.sub.2. Aliquots of cell culture supernatants were harvested .about.36 h post-electroporation, and fresh cultures of ST cells were infected for 1 h at room temperature and subsequently incubated at 37.degree. C. in complete medium.

Recombinant TGEV strains all displayed growth kinetics similar to those of wild-type TGEV generated from the TGEV 1000 infectious construct, with all viruses growing to .about.10.sup.8 PFU/ml in .about.20 h (FIG. 2). Clearly, TGEV ORF 3A and 3B are not required for TGEV replication in vitro.

EXAMPLE 5

Analysis of GFP Expression and RT-PCR to Detect Leader-Containing Sub-Genomic Transcripts

At .about.14-18 h post-electroporation, transfected cultures were analyzed for GFP expression by fluorescent microscopy using an Olympus model inverted microscope. High levels of GFP expression from TGEV-GFP2, but not from the wild-type virus, were evident by fluorescence microscopy. Recombinant TGEV-GFP2 was stable for at least 10 high-titer passages in ST cells, as demonstrated by high levels of GFP expression.

GFP expression was observed in 1% of the cells transfected with TGEV-Rep(AvrII) and TGEV-Rep(EcoNI) RNAs, demonstrating that TGEV ORF 3A, ORF 3B, E, and M were not required for subgenomic mRNA synthesis and GFP expression. Transcripts of TGEV-Rep(AvrII) and TGEV-Rep(EcoNI) treated with RNase A prior to transfection did not result in observable GFP expression. Leader-containing subgenomic transcripts should be present that encode GFP and contain the appropriate deletions that were introduced into the replicon cDNAs. Consequently, total intracellular RNA was harvested from transfected cell cultures (pass 0) with Trizol reagent (Gibco BRL, Carlsbad, Calif.) and from cultures inoculated with pass 0 supernatants (pass 1) at .about.36 h post-infection and was used as a template for reverse transcription-PCR (RT-PCR) using primer sets to detect leader-containing transcripts encoding GFP.

RT reactions were performed using SUPERSCRIPT.TM. II reverse transcriptase for 1 h at 42.degree. C. (250 mM Tris-HCl [pH 8.3], 375 mM KCl, 15 mM MgCl.sub.2, 0.1 M dithiothreitol), as described by the manufacturer (Gibco BRL), prior to PCR amplification using Taq polymerase (Expand Long kit; Roche

Biochemical, Indianapolis, Ind.). To detect leader-containing GFP transcripts in TGEV-GFP2-infected cells, the 5' leader-specific primer (nt 1 to 25) TGEV-L (5'-CAC TAT AGA CTT TTA AAG TAA AGT GAG TGT AGC-3'; SEQ ID NO:18) was used with the 3' primer F:5010(-)(5'-ATT AAG ATG CCG ACA CAC GTC-3'; SEQ ID NO:19), located within ORF 3B at position 24828. To detect leader-containing GFP transcripts derived from replicon RNAs, two different primer sets were utilized. The 5' leader-specific primer, TGEV-L, was used with the 3' primer (-)E5546 (5'-GTT AAT GAC CAT TCC ATT GTC-3'; SEQ ID NO:20), located just downstream of the AvrII site within TGEV E at nucleotide position 25866, to amplify across the PflMI-AvrII deletion. To amplify across the PlfMI-EcoNI deletion, the same 5' leader-specific primer (TGEV-L) was used with the 3' primer M6400(-)(5'-CAA GTG TGT AGA CAA TAG TCC-3'; SEQ ID NO:21), located just downstream of the EcoNI site within TGEV M at nucleotide position 26624. Following 30 cycles of amplification (94.degree. C. for 25 s, 60.degree. C. for 25 s, 68.degree. C. for 90 s), PCR products were separated on agarose gels and visualized by using Dark Reader technology (Clare Chemical Research). All images were digitalized and assembled by using Adobe Photoshop.RTM. 5.5 (Adobe Systems, Inc., San Jose, Calif.).

Appropriately sized amplicons of .about.850 bp were generated from the TGEV-Rep(AvrII)- and TGEV-Rep(EcoNI)-transfected cells (FIG. 3A and FIG. 3B, respectively), corresponding to leader-containing GFP transcripts. Subsequently, the TGEV-Rep(EcoNI) amplicon was isolated and subcloned directly into TOPO.RTM. XL TA cloning vectors (INVITROGEN.TM., Carlsbad, Calif.) as described by the manufacturer. Colonies were isolated on Luria-Bertani plates containing kanamycin (50 .mu.g/ml), and plasmid DNAs were sequenced using an ABI model 377 automated sequencer.

Sequence information confirmed the synthesis of leader-containing GFP transcripts with the PflMI-EcoNI deletion that originated from the ORF 3A TSE. Identical results were seen following TGEV-GFP2 infection, indicating that the 20-nt N gene TSE was silent in this configuration and that the natural ORF 3A TSE was preferentially used in subgenomic mRNA synthesis. Moreover, TGEV iGFP2(ScaI), which has an ATG start codon flanked by the 3A and N gene TSE sites, expressed very low levels of GFP (FIG. 3C). Sequence analysis from over 30 independent clones has confirmed that the GFP subgemonic mRNAs originate from 3A, but not N TSE. This was surprising as: 1) The N gene TSE is the strongest initiator of subgenomic RNAs (Jeong, et al. (1996) Virology 217:311-322; Makino, et al. (1991) J. Virol. 65:6031-6041; Schaad and Baric (1994) J. Virol. 68:8169-8197), and 2) the transcription attenuation model predicts that downstream TSE sites repress expression from upstream sites (Krishnanet al. (1996) Virology 218:400-405; Sawicki and Sawicki (1990) J. Virol. 64:1050-1056). The most likely interpretation of these data is that the N TSE site and its surrounding flanking sequence regulates transcription attenuation of subgenomic RNAs. In the absence of the appropriate flanking sequence, the N TSE is inactive.

In contrast, wild-type TGEV-infected cells yielded multiple amplicons corresponding to leader-containing transcripts carrying TGEV ORF 3A, ORF 3B, E, and M. These transcripts were not detected in TGEV-Rep(AvrII)- and TGEV-Rep(EcoNI)-electroporated cells, respectively, as these genes were completely or partially deleted in the TGEV-Rep constructs (FIG. 1C). Taken together, these data demonstrate the synthesis of subgenomic mRNA and heterologous gene expression from the TGEV-Rep(AvrII) and TGEV-Rep(EcoNI) subgenomic replicon RNAs.

EXAMPLE 6

Analysis of PRRSV GP5 Heterologous Expression

Previous studies have demonstrated that the PRRSV M protein accumulates in the ER of infected cells where it forms disulfide-linked heterodimers with GP5. Heterodimer formation may be critical in eliciting neutralizing antibody against conformational epitopes (Balasuriya, et al. (2000) J. Virol. 74:10623-30). Using the TGEV 3F subclone (FIG. 4A), GFP was removed by ClaI/PflMI digestion and replaced with GP5 of PRRSV to create icTGEV PRSS GP5 recombinant viruses (FIG. 4A). Recombinant viruses expressed PRRSV GP5 antigen as determined by Fluorescent Antibody analysis and by RT-PCR amplification of the gene (FIG. 4B) using primer pairs within the TGEV leader and PRRSV PG5. Leader-primed GP5 PCR amplicon should be about 750 bp (*; lanes 2 and 3), note the smaller PCR amplicon which likely represents cryptic TSE starts (**).

EXAMPLE 7

Gene Order Mutants and Transcription

The results provided above suggest that flanking sequences enhance transcription from the N TSE element. To test this hypothesis, GFP was replaced with the TGEV N gene and N TSE site as shown in FIG. 5. TGEV recombinant viruses were isolated that contained two copies of the N gene (TGEV 2N) as well as TGEV SNEM rearranged viruses that lack the "natural N orientation, and express N from the ORF3 position (FIG. 5). TGEV 2N recombinant viruses were viable and replicated efficiently in ST cells(10.sup.7), demonstrating that gene duplication does not significantly interfere with TGEV replication (FIG. 6). Stability of the TGEV 2N constructs has not yet been studied (Beck and Dawson (1990) Virology 177:462-469). In contrast, the TGEV SNEM gene order mutant TGEV SNEMp4A, purified at passage 4 after the initial transfection, were not robust, only replicating to .about.9.0.times.10.sup.4 PFU/ml. After 9 serial passages of the initial transfection progeny, however, TGEV SNEM1 and 4 viruses were isolated that replicated to titers of .about.10.sup.6 PFU/ml, and each retained N gene expression from the ORF3 position (FIG. 7 and FIG. 8). Sequence analysis has indicated that about 85% of the N gene leader-containing transcripts initiated from the 3A TSE site in TGEV SNEM1, TGEV SNEM4, TGEV 2N-1(PflMI) and TGEV 2N(ScaI) viruses. However, about 15% of the leader-containing transcripts initiated from the N TSE site, supporting the hypothesis that flanking sequences effect N TSE function (FIG. 7)(Alonso, et al (2002) J. Virol. 76:1293-308). These data demonstrate that gene order mutants of TGEV are viable, similar to results described for VSV (Ball, et al. (1999) J. Virol. 73:4705-4712)

To test the hypothesis that compensatory evolution was restoring SNEM virus fitness, TGEV SNEM1 and SNEM4 were serially-passaged 15 times in ST cells. Plaque purified viruses (TGEV SNEM1p15A and TGEV SNEM4p15B) replicated to high .about.10.sup.7 to 10.sup.8 PFU/ml, respectively within about 24 hrs postinfection (FIG. 6). Cultures of ST cells were infected and the "TGEV F fragment" of the TGEV SNEM1 and 4 and revertant viruses were cloned and sequenced (see FIG. 8). SNEM virus fitness was not recovered by recombination events that restored the natural gene order. In contrast to the parental replication impaired TGEV SNEMp4A virus, residual ORF 3B sequences were deleted in TGEV SNEM1 (nucleotides 25,287-25,832) and SNEM 4 (25,197-25,833). As the E TSE is located at position 25,813-25,819 (Almazan, et al. (2000) Proc. Natl. Acad. Sci. USA 97:5516-5521), it is apparently not needed for transcription of subgenomic mRNAs encoding the E protein--an essential gene for virion assembly (Fischer, et al. (1998) J. Virol. 72:7885-7894; Vennema, et al. (1996) EMBO J. 15:2020-2028). In TGEV SNEM1 viruses, a related TSE motif ACAAAAC (SEQ ID NO:13), is located at position 25,275-282 and may serve as an E TSE site. However, no obvious E TSE sites are present in the TGEV SNEM4 viruses. It is hypothesized that these deletions have enhanced TGEV SNEM1 and 4 replication by creating a new E transcriptional regulatory sequence (TRS) and thereby, altering transcription of both the TGEV N and E subgenomic RNAs. Importantly, these findings provide additional support for the hypothesis that the core TSE motif ACTAAAC (SEQ ID NO:1), is a junction site and that other flanking sequences function as regulatory sequences of transcription. The TGEV SNEM1p15A and SNEM4p15B revertants have retained the ORF 3B deletions, and also contain different sets of replacement mutations in the E and M glycoproteins. It is not clear how these changes may enhance virus replication or whether additional mutations may be encoded outside of the F fragment. For vaccine purposes, these data indicate that robust gene order rearranged Coronaviruses can be assembled and used as safe heterologous vaccines in swine and other vertebrates.

EXAMPLE 8

Assembly of TGEV Replicons Encoding GFP

The data presented herein demonstrate that an ORF 3A deletion is not detrimental to stable replication and passage of recombinant TGEV expressing GFP. Consequently, replicon constructs were generated by deleting the E and M structural genes from the previously constructed FiGFP2(PflMI) F fragment (FIG. 1C).

Serial deletions within the TGEV structural gene region were generated from the unique PflMI site at the very 3'

end of the GFP gene and extended for various distances toward the 3' end of the genome (FIG. 1C). In the first construct (pFiGFP2-AvrII), TGEV ORF 3B and the very 5' end of the E gene (first 10 nt), including the E gene TSE and ATG start codon, were removed by PflMI-AvrII digestion. After the digestion, the plasmid was treated with T4 DNA polymerase under conditions in which the 5'.fwdarw.3' exonuclease activity generated blunt ends (according to the manufacturer's directions; New England BioLabs) and religated using T4 DNA ligase. The result was an .about.800-nt deletion from pFiGFP2(PflMI).

In the second construct (pFiGFP2-EcoNI), ORF 3B, E, and the 5'-most 508 nt of the M gene, including the M gene TSE, were removed by PflMI-EcoNI digestion, treated with T4 DNA polymerase to generate blunt ends, and religated using T4 DNA ligase. The result was an .about.1.5-kb deletion.

The unique AvrII site is located at nucleotide position 25866 within the E protein gene, and the unique EcoNI site is located at nucleotide position 26624 within the M protein gene (Almazan, et al. (2000) Proc. Natl. Acad. Sci. USA 97:5516-5521). Clones containing each of the deletions were identified by restriction digestion analysis and confirmed by DNA sequencing using an ABI model 377 automated sequencer. The new TGEV F fragments (FiGFP2-AvrII and FiGFP2-EcoNI) were subsequently utilized in the assembly of full-length TGEV replicon constructs (FIG. 1C).

EXAMPLE 9

Replication Competence of TGEV Replicon RNAs

TGEV-Rep(AvrII) lacks all of ORF 3B and a portion of the E gene and therefore should not produce infectious virions. Successful assembly of infectious TGEV from this replicon should be prevented on at least two levels. First, the E gene TSE and flanking sequences have been deleted in this replicon, which should preclude the synthesis of E gene subgenomic mRNA transcripts. Secondly, the E gene start codon has been deleted, and the next possible ATG start codon is out of the E gene reading frame at nucleotide position 25888 and would potentially encode an irrelevant 14-amino-acid protein. However, one possible in-frame E gene start codon is located 33 bp downstream of the PflMI-AvrII deletion, at nucleotide position 25899, and expression from this site would result in a truncated E protein, with about a 17% (14 of 83 amino acids) deletion from the N-terminus, including residues within a putative membrane anchor. Although unlikely, the expression of a biologically active, truncated E protein may result via read-through from other TGEV mRNAs or from cryptic TSE sites that drive expression of a subgenomic mRNA encoding the E protein, allowing for the assembly of infectious virions. This may be unlikely, as cryptic subgenomic leader-containing E transcripts were not detected by RT-PCR that would encode this E protein truncation. To address whether small amounts of truncated E are produced which function in virus assembly and release, aliquots of cell culture supernatants were harvested .about.36 h post-electroporation and passed onto fresh cultures of ST cells (pass 1). By RT-PCR, there was no evidence of virus replication. In addition, virus-induced cytopathology and GFP expression were not apparent in these cultures.

EXAMPLE 10

Recombinant VEE Replicon Construct

Previous data indicate that the PflMI-AvrII deletion prevented E protein function and the assembly of infectious virus. Consequently, E protein provided in trans should complement the E gene deletion and result in infectious TGEV VRPs. The VEE replicon system has been used previously for the high-level expression of a number of heterologous genes (Balasuriya, et al. (2000) J. Virol. 74:10623-10630; Caley, et al. (1997) J. Virol. 71:3031-3038; Hevey, et al. (1998) Virology 251:28-37; Pushko, et al. (2000) Vaccine 19:142-153; Pushko, et al. (1997) Virology 239:389-401; Schultz-Cherry, et al. (2000) Virology 278:55-59) and was used as an efficient means for expressing the TGEV E protein in trans. It was hypothesized that VEE VRPs expressing TGEV E would supply sufficient concentrations of E protein in trans to allow for efficient assembly and release of packaged TGEV-Rep(AvrII) VRPs (FIG. 9). To determine the effect of VEE VRPs on TGEV replication, cultures of ST cells were either infected with wild-type TGEV alone or coinfected with VEE VRPs containing a G1 VEE-NCFL

capsid gene (Harrington, et al. (2002) J. Virol. 76:730-742) and wild-type TGEV. Progeny TGEV virions were harvested at different times post-infection and quantified by plaque assay in ST cells (FIG. 10). Clearly, the TGEV growth rate was not adversely affected by co-infection with VEE VRPs. Similar results have been shown with another alpha-virus, Sindbis virus, and the murine coronavirus mouse hepatitis virus (MHV) (Baric, et al. (1999) J. Virol. 73:638-649).

The TGEV E gene was inserted into the VEE replicon vector pVR21, kindly provided by Nancy Davis and Robert Johnston (Balasuriya, et al. (2000) J. Virol. 74:10623-10630). Using overlapping extension PCR, the TGEV E gene was inserted just downstream of the subgenomic 26S promoter within the multiple cloning site of pVR21. Using the Expand Long Template PCR system (Roche Molecular Biochemicals), the TGEV (Purdue strain) E gene was amplified from the TGEV F fragment by 30 cycles of PCR (94.degree. C. for 25 s, 60.degree. C. for 25 s, 72.degree. C. for 1 min) by u the TGEV E(V+) 5' primer (5'-AGT CTA GTC CGC CAA GAT GAC GTT TCC TAG GGC ATT G-3'; SEQ ID NO:22) and the AscI site-containing TGEV E(V-) 3' primer (5'-GGC GCG CCT CAA GCA AGG AGT GCT CCA TC-3'; SEQ ID NO:23). In addition, a segment of the pVR21 vector containing a unique SwaI site followed by the 26S subgenomic promoter was amplified by PCR by using the 6198V primer (5'-GCA AAG CTG CGC AGC TTT CC; SEQ ID NO:24) with the (-)7564V primer (5'-CAT CTT GGC GGA CTA GAC TAT GTC GTA GTC CAT TCA GGT TAG CCG; SEQ ID NO:25). Appropriately-sized amplicons were isolated on agarose gels and extracted as previously described (Yount, et al. (2000) J. Virol. 74:10600-10611).

The 5'-most 19 nt of primer (-)7546V were complementary to the 5'-most 19 nt of the 5' TGEV E(V) primer, allowing for the adjoining of the two amplicons by overlapping PCR. Using the Expand Long Template PCR system, reactions were performed and consisted of 30 cycles of 94.degree. C. for 20 s, 58.degree. C. for 30 s, and 68.degree. C. for 2 min, with the first 5 cycles done in the absence of primers. The resulting amplicon, containing unique SwaI and AscI restriction sites at its 5' and 3' ends, respectively, was isolated and purified as previously described. Following AscI and SwaI restriction digest (unique to both the TGEV E amplicon and pVR21), the TGEV E gene was inserted into the pVR21 vector. The resulting recombinant VEE replicon vector (pVR21-E1) was cloned, and the sequence was confirmed using an ABI model 377 automated sequencer. pVR21-E1 was subsequently used for the production of VEE VRPs expressing the TGEV E protein [VEE-TGEV(E)].

A bipartite helper system consisting of two helper RNAs derived from the V3014.DELTA.520-7505 monopartite helper was used for the construction of VEE replicon particles (Pushko, et al. (2000) Vaccine 19:142-153). These helper RNAs express the individual capsid and glycoprotein genes of VEE, thereby supplying the structural genes in trans.

EXAMPLE 11

Recombinant VEE VRP Production

The recombinant VEE replicon construct (pVR21-E1) was linearized at a site downstream of the VEE cDNA sequence by NotI digestion, and T7-capped runoff transcripts were generated in vitro by using the T7 mMessage mMachine.TM. kit as described by the manufacturer (Ambion). Recombinant VEE replicon and helper RNAs were co-electroporated into BHK cells and incubated at 37.degree. C. in 5% CO.sub.2 for .about.24 to 27 h. Cell culture supernatants were harvested and clarified by centrifugation at 12,000.times.g for 15 min. Recombinant VEE VRPs (VEE-TGEV[E]) were partially purified, concentrated, and resuspended in PBS as previously described (Davis, et al. (2000) J. Virol. 74:371-378). Although we were unable to quantitatively identify the presence of VEE-TGEV(E) VRPs due to our lack of anti-E antibody, a qualitative analysis was performed. BHK cells were infected with purified VEE-TGEV(E) VRPs for 1 h at room temperature. VRP titers were high as cytopathic effects were evident in 100% of the transfected cultures, suggesting titers of >10.sup.8 VRP/ml, and transcripts encoding TGEV E were present in infected cells as detected by RT-PCR amplification of leader-containing transcripts.

EXAMPLE 12

Packaging of TGEV Replicon RNA

Two methods were used to supply TGEV E in trans, allowing for the packaging of TGEV-Rep(AvrII) replicon RNA. In the first method, TGEV-Rep(AvrII) replicon RNA and the helper RNA derived from pVR21-E1 were co-electroporated into BHK cells. In the second method, BHK cells were first electroporated with in vitro-transcribed TGEV-Rep(AvrII) RNA (pass 0), seeded onto 75-cm.sup.2 flasks of ST cells, and at 3 h post-electroporation, subsequently infected with recombinant VEE-TGEV(E) VRPs for 1 h at room temperature. Cultures were visualized for GFP expression by fluorescent microscopy at .about.18 h post-electroporation. In both methods, GFP expression was evident by fluorescent microscopy, demonstrating the subgenomic transcription and heterologous gene expression from the TGEV-Rep(AvrII) genome in the presence of VEE replicon RNAs. Conversely, passage of supernatants from cells tranfected with TGEV-Rep(AvrII) transcripts without expression of the E protein in trans did not result in detectable GFP expression.

Cell culture supernatants were harvested .about.36 h post-transfection and undiluted aliquots were used to inoculate fresh cultures of ST cells cultures (75-cm.sup.2 flasks) (pass 1) for 1 h at room temperature to determine if the TGEV-Rep(AvrII) replicon RNA had been packaged into TGEV VRPs. Successful packaging and passing of TGEV-Rep(AvrII) replicon RNA were determined by GFP expression, and RT-PCR analysis was performed to detect leader-containing GFP transcripts, as described above. By .about.18 h post-infection, GFP expression was observed in these pass 1 cultures, confirming that replicon RNAs had been packaged into infectious TGEV VRPs. However, TGEV VRP titers were low, estimated to be 10.sup.3 to 10.sup.5 infectious units/ml by fluorescent microscopy, depending on the experiment. TGEV VRPs should express leader-containing subgenomic mRNAs encoding GFP and the various downstream ORFs, including M and N. Following TGEV VRP infection, intracellular RNA was isolated and subjected to RT-PCR by using primer pairs in the leader RNA and downstream of the GFP, M, and N genes. For this RT-PCR, primer TGEV-L 5' was used in conjunction with the 3' primers TGEV-Mg (5'-AGA AGT TTA GTT ATA CCA TAG GCC TTT ATA CCA TAT GTA ATA ATT TTT CTT GCT CAC TC-3'; SEQ ID NO:26), located at position 26870 within the M gene, and TGEV-Ng (5'-CCA CGC TTT GGT TTA GTT CGT TAC CTC ATC AAT TAT CTC-3'; SEQ ID NO:27), located at position 28038 within the N gene. Briefly, RT reactions were performed by using Superscript.TM. II reverse transcriptase for 1 h at 42.degree. C. (250 mM Tris-HCl [pH 8.3], 375 mM KCl, 15 mM MgCl.sub.2, 0.1 M dithiothreitol), as described by the manufacturer (Gibco BRL), prior to 30 cycles of PCR amplification using Taq polymerase (Expand Long kit; Roche Biochemical) (94.degree. C. for 25 s, 58.degree. C. for 25 s, 68.degree. C. for 1 min and 40 s). PCR products were separated on agarose gels, cloned, and sequenced as previously described.

As in the previous TGEV-Rep(AvrII) experiments, a leader-containing GFP amplicon of .about.850 bp was generated (FIG. 11A) and sequenced to confirm the presence of leader-containing GFP transcripts with the PflMI-AvrII deletion. Leader-containing subgenomic transcripts were also detected that contained the TGEV M and N genes, .about.900 bp and .about.1.2 kb, respectively (FIG. 11B), demonstrating that transcripts for at least two of the structural genes were expressed in TGEV VRP-infected cells. Larger leader-containing amplicons were also observed and likely corresponded to cryptic start sites noted within GFP as well as the larger GFP leader-containing amplicons (FIG. 11B). These data demonstrate the replication competence and heterologous gene expression from packaged TGEV-Rep(AvrII) RNAs.

EXAMPLE 13

TGEV Replicon Particles Function as Single-Hit Virus Vectors

An important aspect of viral replicon particle systems, in terms of future use as an expression vector for vaccine development, is the lack of recombinant virus production. It is possible that mutations may evolve which restore E protein expression and function or recombinant TGEVs emerge following mixed TGEV-Rep(AvrII) and VEE-TGEV(E) infection. To conclusively demonstrate the lack of recombinant virus production from the E deletion replicon RNA, 60-mm.sup.2 cultures of ST cells were infected for 1 h at room temperature with TGEV VRPs obtained from previous TGEV-Rep(AvrII) packaging experiments (clarified and concentrated by high-speed

centrifugation as previously described (Davis, et al. (2000) J. Virol. 74:371-378), overlaid with fresh media, incubated at 37.degree. C., and subsequently examined over a 72-h time period for GFP expression by fluorescent microscopy as well as virus production by plaque assay in ST cells. Under identical conditions, supernatants obtained from cell cultures transfected with TGEV-GFP2 transcripts were passaged onto fresh ST cells and examined for virus replication by GFP expression, for cytopathic effects, and by plaque assay, as previously described.

Expansion of GFP expression was clearly observed in TGEV-GFP2-infected cells while no expansion was noted in TGEV VRP-infected cells. In fact, GFP expression in these TGEV VRP-infected cells eventually decreased after the 24-h time point and eventually disappeared. Additionally, infectious TGEV particles were not detected by plaque assay in TGEV VRP-infected cultures during this same 72-h period (FIG. 12), while infectious TGEV-GFP2 virus rapidly reached titers of 2.times.10.sup.6 PFU/ml by 48 h post-infection under identical conditions. These data clearly demonstrate the lack of revertant wild-type and recombinant virus production from the TGEV-Rep(AvrII) VRP stocks.

The foregoing examples are illustrative of the present invention, and are not to be construed as limiting thereof. The invention is described by the following claims, with equivalents of the claims to be included therein.

SEQUENCE LISTINGS

1

27 1 7 DNA Transmissible Gastroenteritis Virus 1 actaaac 7 2 160 DNA Transmissible Gastroenteritis Virus 2 gtccattaaa tgttaattct atcatcygct ataatagcag ttgtttctgc tagagaattt 60 tgttaaggat gatgaataaa gtctttaaga actaaactta cgagtcatta caggtcctgt 120 atcgattggt ataactaaac ttctaaatgg tgagcaaggg 160 3 154 DNA Transmissible Gastroenteritis Virus 3 tagccttgtg ctagattttg tcttcggaca ccaactcgaa ctaaacttac gagtcattac 60 aggtcctgta tggacattgt caaatccatt tacacatccg tagatgctgt acttgacgaa 120 cttgattgtg catactttgc tgtaactctt aaag 154 4 156 DNA Transmissible Gastroenteritis Virus 4 tagccttgtg ctagattttg tcttcggaca ccaactcgaa ctaaacttac gagtcattac 60 aggtcctgta tcgatatggt ataactaaac ttctaaatgg tgagcaaggg cgaggacgtg 120 ttcaccgggg tggtgcccat cctggtcgag ctggac 156 5 61 DNA Artificial Sequence Sequence of GFP. 5 atggtgagca agggcgagga gctgttcacc ggggtggtgc ccatcctggt cgagctggac 60 g 61 6 118 DNA Transmissible Gastroenteritis Virus 6 tatctcttct tttactttaa ctagccttgt gctagatttt gtcttcggac accaactcga 60 actaaactta cgagtcatta caggtcctgt atggacattg tcaaatccat ttacacat 118 7 124 DNA Transmissible Gastroenteritis Virus 7 tatctcttct tttactttaa ctagccttgt gctagatttt gtcttcggac accaactcga 60 actaaactta cgagtcatta caggtcctgt attgattggt ataactaaac ttctaaatgg 120 ctaa 124 8 124 DNA Transmissible Gastroenteritis Virus 8 tatctcttct tttactttaa ctagccttgt gctagatttt gtcttcggac accaactcga 60 actaaactta cgagtcatta caggtcctgt atcgattggt ataactaaac ttctaaatgg 120 ctaa 124 9 81 DNA Transmissible Gastroenteritis Virus 9 tatctcttct tttactttaa ctagccttgt gctagatttt gtcttcggac accaactcga 60 actaaacttc taaatggcta a 81 10 81 DNA Transmissible Gastroenteritis Virus 10 tatctcttct tttactttaa cttgccttgt gctagatttt gtcttcggac accaactcga 60 actaaacttc taaatggcta a 81 11 8 DNA Transmissible Gastroenteritis Virus misc_feature (1)..(8) N Gene Sequence. 11 atggccaa 8 12 123 DNA Artificial Sequence Consensus sequence of intergenic sequence. 12 tatctcttct tttactttaa ctagccttgt gctagatttt gtcttcggac accaactcga 60 actaaactta cgagtcatta caggtcctgt atgattggta taactaaact tctaaatggc 120 taa 123 13 7 DNA Transmissible Gastroenteritis Virus 13 acaaaac 7 14 7 DNA Transmissible Gastroenteritis Virus 14 actaaac 7 15 7 DNA Transmissible Gastroenteritis Virus 15 tctaaac 7 16 6 DNA Transmissible Gastroenteritis Virus 16 agtact 6 17 20 DNA Artificial Sequence Synthetic oligonucleotide primer 17 tggtataact aaacttctaa 20 18 33 DNA Artificial Sequence Synthetic oligonucleotide primer 18 cactatagac ttttaaagta aagtgagtgt agc 33 19 21 DNA Artificial Sequence Synthetic oligonucleotide primer 19 attaagatgc cgacacacgt c 21 20 21 DNA Artificial Sequence Synthetic oligonucleotide primer 20 gttaatgacc attccattgt c 21 21 21 DNA Artificial Sequence Synthetic oligonucleotide primer 21 caagtgtgta gacaatagtc c 21 22 37 DNA Artificial Sequence Synthetic oligonucleotide primer 22 agtctagtcc gccaagatga cgtttcctag ggcattg 37 23 29 DNA Artificial Sequence Synthetic oligonucleotide primer 23 ggcgcgcctc aagcaaggag tgctccatc 29 24 20 DNA Artificial Sequence Synthetic oligonucleotide primer 24 gcaaagctgc gcagctttcc 20 25 45 DNA Artificial Sequence Synthetic oligonucleotide primer 25 catcttggcg gactagacta tgtcgtagtc cattcaggtt agccg 45 26 59 DNA Artificial Sequence Synthetic oligonucleotide primer 26 agaagtttag ttataccata ggcctttata ccatatgtaa taatttttct tgctcactc 59 27 39 DNA Artificial

Sequence Synthetic oligonucleotide primer 27 ccacgctttg gtttagttcg ttacctcatc aattatctc 39

* * * * *

EXHIBIT C

Diemiruaya Ogheneakpor Deniran
3155 Rochambeau Avenue,
#9D,
Bronx, New York, 10467
9/16/2021

Ashley Murphy-Rutski
Benefits Coordinator
Museum of Modern Art ("MoMA" or "Museum")
11 West 53rd street
New York, NY 10019

RE: Mandatory COVID-19 Vaccination Policy AND YOUR FAILURE/REFUSAL TO PROVIDE PROOF OF DELEGATION OF AUTHORITY TO ACT FROM THE CITY OF NEW YORK & THE STATE OF NEW YORK AS INDICATION OF YOUR INTENT TO become a tort feasor in your capacity as a *state actor* as defined in 42 U.S. Code § 1983, and your capricious violation of TITLE 18, U.S.C., SECTION 242.

ATTENTION: Ashley Murphy-Rutski

NUNC PRO TUNC CONDITIONAL ACCEPTANCE FOR VALUE FOR PROOF OF CLAIM & irrevocable admission of liability, tender of certified surety related to the entire contents of your contumacious e-mail response WHEREIN YOU SAID AS FOLLOWS IN YOUR E-MAIL RESPONSE DATED 9/13/2021 : 3:47PM

" *We have reviewed your request for a reasonable accommodation based on your stated religious beliefs. Without assessing whether your religious beliefs actually prohibit vaccination, we are unable to accommodate your request to work at MoMA while unvaccinated. We are willing to provide a 30-day unpaid leave of absence, starting September 11, 2021.*" [SEE attached, emphasis is ours]

Diemiruaya Deniran now says as follows:

1. PLEASE TAKE NOTICE that as cited above in your e-mail response to our earlier NOTICE/RESPONSE:
2. WE FIND that, you have contumaciously and disingenuously mischaracterized the FACTS OF LAW WE BROUGHT TO YOUR AWARENESS THAT EXPRESSLY PROHIBIT YOUR ACTS OF FORCIBLE TERMINATION , AND IN SO DOING HAVE agreed and are willing to violate the dictates of 42 U.S. Code § 1983, and TITLE 18, U.S.C., SECTION 242, without protest. WE THEREFORE, FOR THE RECORD, REMIND AGAIN THAT THE SPECIFIC AREAS OF LAW WE CITED THAT PROHIBIT YOUR ACTS HAVE NOTHING TO DO WITH RELIGIOUS BELIEF.
3. WE FURTHER PUT YOU ON NOTICE THAT YOU REFUSED TO PROVIDE PROOF OF DELEGATION OF AUTHORITY FROM THE NEW YORK STATE ASSEMBLY stating that you can ACT on the behalf of CITY OF NEW YORK & STATE OF NEW YORK in a governmental capacity, NOT MINISTERIAL.
4. AND THAT YOU have continued to use threats, coercion, brigandry, under color of law and authority to deny the affiant his means of daily survival NOT BASED ON JOB OUTLINE incompetence, malfeasance

or tardiness, but on your overzealous declared intent to IGNORE AND VIOLATE established principles of law as already copiously outlined in our earlier NOTICE TO YOU.

5. AS SUCH, PLEASE TAKE NOTICE, as per the forgoing that you have agreed to be held absolutely PERSONALLY liable for ACT OF UNLAWFUL CONVERSION of legitimate work hours into " unpaid leave of absence" (your words), through your unconstitutional acts under color of law and authority. [SEE ATTACHED OUR PREVIOUS NOTICES TO YOU & YOURS]

6. **PLEASE TAKE NOTICE** THAT YOUR REFUSAL TO provide the demanded responses will be proof that you intended to be deemed a tort feasor in your capacity as a *state actor* as defined in 42 U.S. Code § 1983, including but not limited to other liabilities as found in TITLE 18, U.S.C., SECTION 242, and we will avail ourselves of all equitable remedies accorded to us in this matter. FIND BELOW THE SUPREME LAWS OF THE LAND THAT PROHIBIT YOUR CAPRICIOUS & WANTON ACTS:

7. Unconstitutional conditions case law often references the existence of varying degrees of coercion. According to that body of law, MOMA cannot impair Diemiruaya Deniran's right to refuse medical care through

subtle forms of coercion any more than it could through an explicit mandate. See, e.g., *Koontz v. St. Johns River Water Mgmt. Dist.*, 570 U.S. 595 (2013)

8. ("[U]nconstitutional conditions doctrine forbids burdening the Constitution's enumerated rights by coercively withholding benefits from those who exercise them"); Memorial Hosp. v. Maricopa Cty., 415 U.S. 250 (1974) ("[An] overarching principle, known as the unconstitutional conditions doctrine … vindicates the Constitution's enumerated rights by preventing the government from coercing people into giving them up").

9. The government "may not deny a benefit to a person on a basis that infringes his constitutionally protected interests"); *Wieman v. Updegraff*, 344 U.S. 183, 192 (1952)

10. The United States Constitution and federal laws are the "Supreme Law of the Land" and supersede the constitutions and laws of any state. U.S. Const. art. VI, cl. 2.

11. "State law is pre-empted to the extent that it actually conflicts with federal law." English v. General Elec. Co., 496 U.S. 72, 79 (1990)

General acquiescence or non-response by Museum of Modern Art ("MoMA" or "Museum") to provide the above **'Proofs of Claim'** will constitute your agreement and formal acceptance. You will have by your non-response FAILED to state a

claim upon which relief can be granted otherwise shall operate as general acquiescence relative to this presentment. You will have admitted there is no valid Claim of Action arising FROM YOUR INTENTION TO IMPLEMENT a coercive, intrusive and unconstitutional MANDATE, and in so doing you admit liability and surety to the affiant IF YOU INDEED CARRY out your threat of forcible termination of his current employment.

Due to the time sensitive nature of this private matter, under necessity, you are to respond with **'Proof of Claim'** within 24 hours by return service by certified-priority-return-mail to the undersigned's address .

Should you fail or refuse by non-response to provide **'Proof of Claim'** within the time specified in this private matter, general acquiescence and acceptance of full liabilities will be taken on your part as formally exercised (performed) pursuant to your silence.

This agreement shall have the implication of an instrument under seal.

Sincerely,

Without Prejudice

Authorized Representative, Attorney-In-Fact

By: _____

Diemirdaya Ogheneakpor Deniran
Secured Party Creditor
9/16/2021

MoMA

Deniran, Diemiruaya <diemiruaya_deniran@moma.org>

NYC Vaccine Mandate

1 message

Murphy, Ashley <ashley_murphy@moma.org> Mon, Sep 13, 2021 at 3:47 PM
To: "Deniran, Diemiruaya" <diemiruaya_deniran@moma.org>
Cc: Daniel Platt <daniel_platt@moma.org>, Tyrone Wyllie <tyrone_wyllie@moma.org>, Caroline Clements <caroline_clements@moma.org>

In accordance with the Key to NYC Mandate and MoMA's Vaccination Policy based on our safety assessment, all employees must be vaccinated to enter the Museum. In order for you to perform the essential functions of your job, you must be physically present on the premises of the Museum.

We have reviewed your request for a reasonable accommodation based on your stated religious beliefs. Without assessing whether your religious beliefs actually prohibit vaccination, we are unable to accommodate your request to work at MoMA while unvaccinated. We are willing to provide a 30-day unpaid leave of absence, starting September 11, 2021.

While you remain unvaccinated, you may not report to work and will not be paid.

Please be in touch if you have questions or if you elect to receive one of the COVID-19 vaccines.

Ashley Murphy-Rutski
Benefits Coordinator
The Museum of Modern Art
ashley_murphy@moma.org
Phone (212) 708-9839
Fax (212) 333-1107

EXHIBIT D

Diemiruaya Ogheneakpor Deniran
3155 Rochambeau Avenue,
#9D,
Bronx, New York, 10467
9/10/2021

Caroline Clements
Benefits Manager
Museum of Modern Art ("MoMA" or "Museum")
11 West 53rd street
New York, NY 10019

RE: Mandatory COVID-19 Vaccination Policy AND YOUR FAILURE/REFUSAL TO PROVIDE PROOF OF DELEGATION OF AUTHORITY TO ACT FROM THE CITY OF NEW YORK & THE STATE OF NEW YORK AS INDICATION OF YOUR INTENT TO become a tort feasor in your capacity as a *state actor* as defined in 42 U.S. Code § 1983, and your capricious violation of TITLE 18, U.S.C., SECTION 242.

TO CAROLINE CLEMENTS:

NUNC PRO TUNC CONDITIONAL ACCEPTANCE FOR VALUE FOR PROOF OF CLAIM & irrevocable admission of liability, tender of certified surety related to the entire contents of your contumacious e-mail response WHEREIN YOU SAID AS FOLLOWS IN YOUR E-MAIL RESPONSE DATED 9/9/2021 :

" Regardless of whether your accommodation request is approved, no employee who isn't vaccinated will be allowed onsite after tomorrow. Even if we're able to approve your request, the accommodation would be an unpaid leave of absence. This means that if you do not show us proof that you've received the first dose of a vaccine by tomorrow, you will be unpaid beginning on Saturday." [SEE attached]

STATE OF NEW YORK):

COUNTY OF NEW YORK):

Diemiruaya Deniran now says as follows:

1. PLEASE TAKE NOTICE that as cited above in your e-mail response to our earlier NOTICE/RESPONSE:

2. WE FIND that, you have contumaciously agreed and are willing to violate the dictates of 42 U.S. Code § 1983, and TITLE 18, U.S.C., SECTION 242,

3. THAT YOU REFUSED TO PROVIDE PROOF OF DELEGATION OF AUTHORITY FROM THE NEW YORK STATE ASSEMBLY stating that you can ACT on the behalf of CITY OF NEW YORK & STATE OF NEW YORK in a governmental capacity, NOT MINISTERIAL.

4. AND YET YOU have continued to use threats, coercion, brigandry, under color of law and authority to deny the affiant his means of daily survival NOT BASED ON JOB OUTLINE incompetence, malfeasance or tardiness, but on your overzealous declared intent to IGNORE AND VIOLATE established principles of law as already copiously outlined in our earlier NOTICE TO YOU.

5. AS SUCH, PLEASE TAKE NOTICE, as per the forgoing that you have agreed to be held absolutely liable for ACT OF UNLAWFUL

CONVERSION of legitimate work hours into " unpaid leave of absence" (your words)

6. **PLEASE TAKE NOTICE** THAT YOUR REFUSAL TO provide the demanded responses will be proof that you intended to be deemed a tort feasor in your capacity as a *state actor* as defined in 42 U.S. Code § 1983, including but not limited to other liabilities as found in TITLE 18, U.S.C., SECTION 242.

7. Unconstitutional conditions case law often references the existence of varying degrees of coercion. According to that body of law, MOMA cannot impair Diemiruaya Deniran's right to refuse medical care through subtle forms of coercion any more than it could through an explicit mandate. See, e.g., *Koontz v. St. Johns River Water Mgmt. Dist.*, 570 U.S. 595 (2013)

8. ("[U]nconstitutional conditions doctrine forbids burdening the Constitution's enumerated rights by coercively withholding benefits from those who exercise them"); Memorial Hosp. v. Maricopa Cty., 415 U.S. 250 (1974) ("[An] overarching principle, known as the unconstitutional conditions doctrine ... vindicates the Constitution's enumerated rights by preventing the government from coercing people into giving them up").

9. The government "may not deny a benefit to a person on a basis that infringes his constitutionally protected interests"); *Wieman v. Updegraff*, 344 U.S. 183, 192 (1952)

10. The United States Constitution and federal laws are the "Supreme Law of the Land" and supersede the constitutions and laws of any state. U.S. Const. art. VI, cl. 2.

11. "State law is pre-empted to the extent that it actually conflicts with federal law." English v. General Elec. Co., 496 U.S. 72, 79 (1990)

General acquiescence or non-response by Museum of Modern Art ("MoMA" or "Museum") to provide the above **'Proofs of Claim'** will constitute your agreement and formal acceptance. You will have by your non-response FAILED to state a claim upon which relief can be granted otherwise shall operate as general acquiescence relative to this presentment. You will have admitted there is no valid Claim of Action arising FROM YOUR INTENTION TO IMPLEMENT a coercive, intrusive and unconstitutional MANDATE, and in so doing you admit liability and surety to the affiant IF YOU INDEED CARRY out your threat of forcible termination of his current employment.

Due to the time sensitive nature of this private matter, under necessity, you are to respond with **'Proof of Claim'** within 24 hours by return service by certified-priority-return-mail to the undersigned's address .

Should you fail or refuse by non-response to provide **'Proof of Claim'** within the time specified in this private matter, general acquiescence and acceptance will be taken on your part as formally exercised (performed) pursuant to your silence.

This agreement shall have the implication of an instrument under seal.

Sincerely,

Without Prejudice

Authorized Representative, Attorney-In-Fact

By: _____

Diemiruaya Ogheneakpor Deniran
Secured Party Creditor

NEW YORK NOTARY PUBLIC _____

MY COMMISSION EXPIRES

MoMA

Deniran, Diemiruaya <diemiruaya_deniran@moma.org>

Mandatory COVID-19 Vaccination Policy

Clements, Caroline <caroline_clements@moma.org> Thu, Sep 9, 2021 at 1:05 PM
To: "Deniran, Diemiruaya" <diemiruaya_deniran@moma.org>
Cc: "Murphy, Ashley" <ashley_murphy@moma.org>

Thank you for sending this; we're currently evaluating it.

Regardless of whether your accommodation request is approved, no employee who isn't vaccinated will be allowed onsite after tomorrow. Even if we're able to approve your request, the accommodation would be an unpaid leave of absence. This means that if you do not show us proof that you've received the first dose of a vaccine by tomorrow, you will be unpaid beginning on Saturday..

[Quoted text hidden]

--

Caroline Clements
Benefits Manager
The Museum of Modern Art
caroline_clements@moma.org
Phone (212) 708-9455
Fax (212) 333-1107

MoMA is now open with new hours and safety protocols. We look forward to seeing you at the Museum. For more information, please visit our website at moma.org.

MoMA

Deniran, Diemiruaya <diemiruaya_deniran@moma.org>

Mandatory COVID-19 Vaccination Policy

Clements, Caroline <caroline_clements@moma.org> Thu, Sep 9, 2021 at 1:05 PM
To: "Deniran, Diemiruaya" <diemiruaya_deniran@moma.org>
Cc: "Murphy, Ashley" <ashley_murphy@moma.org>

Thank you for sending this; we're currently evaluating it.

Regardless of whether your accommodation request is approved, no employee who isn't vaccinated will be allowed onsite after tomorrow. Even if we're able to approve your request, the accommodation would be an unpaid leave of absence. This means that if you do not show us proof that you've received the first dose of a vaccine by tomorrow, you will be unpaid beginning on Saturday.

On Wed, Sep 8, 2021 at 4:36 PM Deniran, Diemiruaya <diemiruaya_deniran@moma.org> wrote:

> Greetings Caroline,
>
> I attempted several times to attach the documents to the **Key to NYC Pass - Vaccine Mandate** but each time I received the following message: ***"All your files failed to upload. Please retry or remove the failed files. You may also add new files."*** I have attached the documents with this email.
>
> Best regards,
>
> --
> **Diemiruaya Deniran**
> Control Room Operator/Assistant Supervisor
> Security Department
>
> The Museum of Modern Art
> 11 West 53 Street
> New York, NY 10019
> (212) 708 9467
> moma.org

--
Caroline Clements
Benefits Manager
The Museum of Modern Art
caroline_clements@moma.org
Phone (212) 708-9455
Fax (212) 333-1107

MoMA is now open with new hours and safety protocols. We look forward to seeing you at the Museum. For more information, please visit our website at moma.org.

EXHIBIT E

Diemiruaya Ogheneakpor Deniran
3155 Rochambeau Avenue,
#9D,
Bronx, New York, 10467
10/1/2021

James Grooms
General Counsel & Secretary at The Museum of Modern Art
11 West 53 Street
New York, NY 10019

RE: Mandatory COVID-19 Vaccination Policy

NUNC PRO TUNC NOTICE OF COUNTER-CLAIMS FOR TORTIOUS INTERFERENCE & WANTON VIOLATION OF THE US CONSTITUTION AND SUPREME LAWS OF THE LAND AS PER YOUR FAILURE & REFUSAL TO PRODUCE YOUR SOURCE OF AUTHORITY TO CAPRICIOUSLY & PREMATURELY VOID, TERMINATE AND ERASE MY PROFESSIONAL CAREER/LIVELIHOOD CAUSING IRREPARABLE DAMAGES AND PRECARIOUS FUTURE. [SEE Cruzan, 497 U.S. at 278; *King v. Rubenstein*, 825 F.3d 206, 222 (4th Cir. 2016)]

STATE OF NEW YORK):

COUNTY OF NEW YORK):

Diemiruaya Deniran now says as follows:

1. WHEREAS, You were properly served NOTICE demanding that you produce for the record the source of your authority to force me to vaccinate and forcefully terminate my job,
2. AND WHEREAS, LET THE RECORD SHOW, I NEVER INTENDED TO VOLUNTARILY ABANDON MY JOB, AS SUCH IT REMAINS A

FACT THAT YOU & YOURS FORCED AND IMPOSED A PREMATURE TERMINATION through your threats and coercion.

3. AND WHEREAS, in the said NOTICE you were made aware of the fact that your so-called enforcement of A coerced and forced vaccine was in violation of the Supreme Law of the Land as detailed in, " The Supreme Court has recognized that the Ninth and Fourteenth Amendments protect an individual's right to privacy. A "forcible injection . . . into a non-consenting person's body represents a substantial interference with that person's liberty[.]" *Washington v. Harper*, 494 U.S. 210, 229 (1990).

4. AND WHEREAS, YOU WERE FURTHER MADE AWARE THAT, your so-called enforcement of A coerced and forced vaccine was in violation of the Supreme Law of the Land as detailed in, THE US CONSTITUTION, protects a person's right to "refus[e] unwanted medical care." Cruzan, 497 U.S. at 278; King v. Rubenstein, 825 F.3d 206, 222 (4th Cir. 2016). AND AS SUCH, pursuant to the Supreme Doctrine found in "*The claim and exercise of a constitutional right cannot be converted into a crime.*" *Miller v. US*, 230 F 486, at 489.]

5. AND WHEREAS, YOU WERE FURTHER MADE AWARE THAT, your so-called enforcement of A coerced and forced vaccine was in violation of the Supreme Law of the Land as detailed in, This right is "so

rooted in our history, tradition, and practice as to require special protection under the Fourteenth Amendment." Washington v. Glucksberg, 521 U.S. 702, 722 n.17 (1997).

6. AND WHEREAS, YOU WERE FURTHER MADE AWARE THAT, your so-called enforcement of A coerced and forced vaccine was in violation of the Supreme Law of the Land as detailed in, "[U]nconstitutional conditions doctrine forbids burdening the Constitution's enumerated rights by coercively withholding benefits from those who exercise them"); *Memorial Hosp. v. Maricopa Cty.*, 415 U.S. 250 (1974) ("[An] overarching principle, known as the unconstitutional conditions doctrine … vindicates the Constitution's enumerated rights by preventing the government from coercing people into giving them up").

7. AND WHEREAS, YOU WERE FURTHER MADE AWARE THAT, your so-called enforcement of A coerced and forced vaccine was in violation of the Supreme Law of the Land as detailed in, The government "may not deny a benefit to a person on a basis that infringes his constitutionally protected interests"); *Wieman v. Updegraff*, 344 U.S. 183, 192 (1952)

8. AND WHEREAS, all of your enumerated intentional violations as detailed in the preceding paragraphs have indisputably qualified you to

, be a liable actor within the framework of you and yours as tort feasors in your capacities as a *state actors* as defined in 42 U.S. Code § 1983, including but not limited to other liabilities as found in TITLE 18, U.S.C., SECTION 242.

9. WHEREFORE, as per the specifically outlined intentional violations AND YOUR REFUSAL TO ABATE SUCH VIOLATIONS AFTER SUFFICIENT DUE PROCESS NOTICES, you have been judged liable of, the damaged party, herein, to wit, foregoing a protective claim of lien in the amount of $2,000,000 (Two Million US Dollars) FOR YOUR CONTUMACIOUS VIOLATIONS OF LAW and placing in jeopardy my future and the futures of all the people who depend on me.

10. The United States Constitution and federal laws are the "Supreme Law of the Land" and supersede the constitutions and laws of any state. U.S. Const. art. VI, cl. 2.

11. "State law is pre-empted to the extent that it actually conflicts with federal law." English v. General Elec. Co., 496 U.S. 72, 79 (1990)

12. AS SUCH, THIS CLAIM OF PROTECTIVE LIEN SHALL REMAIN IRREVOCABLE NUNC PRO TUNC, UNTIL YOU ABATE THE DAMAGES YOU HAVE CAUSED.

This agreement shall have the implication of an instrument under seal.

Sincerely,

Without Prejudice

Authorized Representative, Attorney-In-Fact

By: _____

Diemruaya Ogheneakpor Deniran
Secured Party Creditor

NEW YORK NOTARY PUBLIC

_____

ANJAYKUMAR K. PUNJANI
Notary Public-State of New York
Bronx County, # 01PU6082273
My Commission Expires
October 21, 20

MY COMMISSION EXPIRES

Ashley Murphy-Rutski
Benefits Coordinator
Museum of Modern Art ("MoMA" or "Museum")
11 West 53rd street
New York, NY 10019